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MENU

Official Classification.

Protease types

Classification

Substrates

Protease is the common name for enzymes that catalyze the cleavage of peptide bonds. More formally known as peptidases (short for peptide bond hydrolases), the Enzyme Commision gives them numbers in the 3.4.X.X range. For more details of their classification according to Enzyme Commision rules you can go to the Barrett & Rawlings list. This will give you a frequently updated list of hundreds of proteases with embedded links to their relative data bank entries.

Specificity

The key to the official classification is to consider that each protease is divided into a group according to the chemical mechanism it uses to achieve peptide bond cleavage.

CATALYTIC CLASS	MECHANISM	COMMON EXAMPLES
Serine	Covalent attack of polarized serine side-chain	Trypsin, tPA, subtilisin, CPD-Y, granzymes, HSV PR
Cysteine	Covalent attack of polarized cysteine side-chain	Papain, caspases, calpain, cathepsin B, Polio 3C PR, DPP1
Threonine	Covalent attack of polarized threonine side-chain	Proteasome
Metallo	Water molecule attack polarized by enzyme coordinated Zn	Thermolysin, MMPs, TACE, CPA, TOP
Aspartic	Water molecule attack polarized by enzyme aspartic side-chains	Pepsin, cathepsin D, HIV PR
Unknown	Huh?	Prohead endopeptidase (phage T4)

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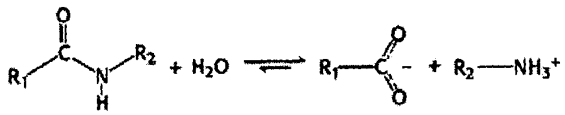
9.1. Proteases: Facilitating a Difficult Reaction

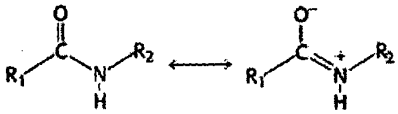
Protein turnover is an important process in living systems (Chapter 23). Proteins that have served their purpose must be degraded so that their constituent amino acids can be recycled for the synthesis of new proteins. Proteins ingested in the diet must be broken down into small peptides and amino acids for absorption in the gut. Furthermore, as described in detail in Chapter 10, proteolytic reactions are important in regulating the activity of certain enzymes and other proteins.

Proteases cleave proteins by a hydrolysis reaction—the addition of a molecule of water to a peptide bond:



Although the hydrolysis of peptide bonds is thermodynamically favored, such hydrolysis reactions are extremely slow. In the absence of a catalyst, the half-life for the hydrolysis of a typical peptide at neutral pH is estimated to be between 10 and 1000 years. Yet, peptide bonds must be hydrolyzed within milliseconds in some biochemical processes.

The chemical bonding in peptide bonds is responsible for their kinetic stability. Specifically, the resonance structure that accounts for the planarity of a peptide bond (Section 3.2.2) also makes such bonds resistant to hydrolysis. This resonance structure endows the peptide bond with partial double-bond character:



The carbon-nitrogen bond is strengthened by its double-bond character, and the carbonyl carbon atom is less electrophilic and less susceptible to nucleophilic attack than are the carbonyl carbon atoms in compounds such as carboxylate esters. Consequently, to promote peptide-bond cleavage, an enzyme must facilitate nucleophilic attack at a normally unreactive carbonyl group.

9.1.1. Chymotrypsin Possesses a Highly Reactive Serine Residue

A number of proteolytic enzymes participate in the breakdown of proteins in the digestive systems of mammals and other organisms. One such enzyme, chymotrypsin, cleaves peptide bonds selectively on the carboxylterminal side of the large hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine, and methionine (Figure 9.1). Chymotrypsin is a good example of the use of *covalent modification* as a catalytic strategy. The enzyme employs a powerful nucleophile to attack the unreactive carbonyl group of the substrate. This nucleophile becomes covalently attached to the

h cb hg e e e fcg c e ch h h

substrate briefly in the course of catalysis.

What is the nucleophile that chymotrypsin employs to attack the substrate carbonyl group? A clue came from the fact that chymotrypsin contains an extraordinarily reactive serine residue. Treatment with organofluorophosphates such as diisopropylphosphofluoridate (DIPF) (Section 8.5.2) was found to inactivate the enzyme irreversibly (Figure 9.2). Despite the fact that the enzyme possesses 28 serine residues, only one, serine 195, was modified, resulting in a total loss of enzyme activity. This *chemical modification reaction* suggested that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin. TOP

9.1.2. Chymotrypsin Action Proceeds in Two Steps Linked by a Covalently Bound Intermediate

Conceptual Insights, Enzyme Kinetics. See the section entitled "Pre-Steady-State Kinetics" in Conceptual Insights module to better understand why a "burst" phase at short reaction times implies the existence of an enzyme-substrate intermediate.

How can we elucidate the role of serine 195 in chymotrypsin action? A study of the enzyme's kinetics provided a second clue to chymotrypsin's catalytic mechanism and the role of serine 195. The kinetics of enzyme action are often easily monitored by having the enzyme act on a substrate analog that forms a colored product. For chymotrypsin, such a *chromogenic substrate* is *N*-acetyl-l-phenylalanine *p*-nitrophenyl ester. This substrate is an ester rather than an amide, but many proteases will also hydrolyze esters. One of the products formed by chymotrypsin's cleavage of this substrate is *p*- nitrophenolate, which has a yellow color (Figure 9.3). Measurements of the absorbance of light revealed the amount of *p*-nitrophenolate being produced.

Under steady-state conditions, the cleavage of this substrate obeys Michaelis-Menten kinetics with a K_M of 20 μM and a k_{cat} of 77 s^{-1}. The initial phase of the reaction was examined by using the stopped-flow method. This technique permits the rapid mixing of enzyme and substrate and allows almost instantaneous monitoring of the reaction. At the beginning of the reaction, this method revealed a "burst" phase during which the colored product was produced rapidly (Figure 9.4). Product was then produced more slowly as the reaction reached the steady state. These results suggest that hydrolysis proceeds in two steps. The burst is observed because, for this substrate, the first step is more rapid than the second step.

The two steps are explained by the reaction of the serine nucleophile with the substrate to form the covalently bound enzyme-substrate intermediate (Figure 9.5). First, the highly reactive serine 195 hydroxyl group attacks the carbonyl group of the substrate to form the acyl-enzyme intermediate, releasing the alcohol *p*-nitrophenol (or an amine if the substrate is an amide rather than an ester). Second, the acyl-enzyme intermediate is hydrolyzed to release the carboxylic acid component of the substrate and regenerate the free enzyme. Thus, *p*-nitrophenolate is produced rapidly on the addition of the substrate as the acyl-enzyme intermediate is formed, but it takes longer for the enzyme to be "reset" by the hydrolysis of the acyl-enzyme intermediate. TOP

9.1.3. Serine is Part of a Catalytic Triad That Also Includes Histidine and Aspartic Acid

Structural Insights, Chymotrypsin: A Serine Protease. Work with interactive molecular models to learn more about the structural bases of active site specificity and reactivity, and some of the ways in which active site residues can be identified.

The determination of the three-dimensional structure of chymotrypsin by David Blow in 1967 was a source of further insight into its mechanism of action. Overall, chymotrypsin is roughly spherical and comprises three polypeptide chains, linked by disulfide bonds. It is synthesized as a single polypeptide, termed chymotrypsinogen, which is activated by the proteolytic cleavage of the polypeptide to yield the three chains. The active site of chymotrypsin, marked by serine 195, lies in a cleft on the surface of the enzyme (Figure 9.6). The structural analysis revealed the chemical basis of the special reactivity of serine 195 (Figure 9.7). The side chain of serine 195 is hydrogen bonded to the imidazole ring of histidine 57. The -NH group of this imidazole ring is, in turn, hydrogen bonded to the carboxylate group of aspartate 102. This constellation of residues is referred to as the *catalytic triad.* How does this arrangement of residues lead to the high reactivity of serine 195? The histidine residue serves to position the serine side chain and to polarize its hydroxyl group. In doing so, the residue acts as a

h cb hg e e e fcg c e ch b b c e

general base catalyst, a hydrogen ion acceptor, because the polarized hydroxyl group of the serine residue is poised for deprotonation. The withdrawal of the proton from the hydroxyl group generates an alkoxide ion, which is a much more powerful nucleophile than an alcohol is. The aspartate residue helps orient the histidine residue and make it a better proton acceptor through electrostatic effects.

These observations suggest a mechanism for peptide hydrolysis (Figure 9.8). After substrate binding (step 1), the reaction begins with the hydroxyl group of serine 195 making a nucleophilic attack on the carbonyl carbon atom of the substrate (step 2). The nucleophilic attack changes the geometry around this carbon atom from trigonal planar to tetrahedral. The inherently unstable *tetrahedral intermediate* formed bears a formal negative charge on the oxygen atom derived from the carbonyl group. This charge is stabilized by interactions with NH groups from the protein in a site termed the *oxyanion hole* (Figure 9.9). These interactions also help stabilize the transition state that precedes the formation of the tetrahedral intermediate. This tetrahedral intermediate then collapses to generate the acyl-enzyme (step 3). This step is facilitated by the transfer of a proton from the positively charged histidine residue to the amino group formed by cleavage of the peptide bond. The amine component is now free to depart from the enzyme (step 4) and is replaced by a water molecule (step 5). The ester group of the acyl-enzyme is now hydrolyzed by a process that is essentially a repeat of steps 2 through 4. The water molecule attacks the carbonyl group while a proton is concomitantly removed by the histidine residue, which now acts as a general acid catalyst, forming a tetrahedral intermediate (step 6). This structure breaks down to form the carboxylic acid product (step 7). Finally, the release of the carboxylic acid product (step 8) readies the enzyme for another round of catalysis.

This mechanism accounts for all characteristics of chymotrypsin action except the observed preference for cleaving the peptide bonds just past residues with large, hydrophobic side chains. Examination of the threedimensional structure of chymotrypsin with substrate analogs and enzyme inhibitors revealed the presence of a deep, relatively hydrophobic pocket, called the S_1 pocket, into which the long, uncharged side chains of residues such as phenylalanine and tryptophan can fit. *The binding of an appropriate side chain into this pocket positions the adjacent peptide bond into the active site for cleavage* (Figure 9.10). The specificity of chymotrypsin depends almost entirely on which amino acid is directly on the amino-terminal side of the peptide bond to be cleaved. Other proteases have more-complex specificity patterns, as illustrated in Figure 9.11. Such enzymes have additional pockets on their surfaces for the recognition of other residues in the substrate. Residues on the amino-terminal side of the scissile bond (the bond to be cleaved) are labeled P_1, P_2, P_3, and so forth, indicating their positions in relation to the scissile bond. Likewise, residues on the carboxyl side of the scissile bond are labeled P_1', P_2', P_3', and so forth. The corresponding sites on the enzyme are referred to as S_1, S_2 or S_1', S_2', and so forth. TOP

9.1.4. Catalytic Triads Are Found in Other Hydrolytic Enzymes

Many other proteins have subsequently been found to contain catalytic triads similar to that discovered in chymotrypsin. Some, such as trypsin and elastase, are obvious homologs of chymotrypsin. The sequences of these proteins are approximately 40% identical with that of chymotrypsin, and their overall structures are nearly the same (Figure 9.12). These proteins operate by mechanisms identical with that of chymotrypsin. However, they have very different substrate specificities. Trypsin cleaves at the peptide bond after residues with long, positively charged side chains—namely, arginine and lysine—whereas elastase cleaves at the peptide bond after amino acids with small side chains—such as alanine and serine. Comparison of the S_1 pockets of these enzymes reveals the basis of the specificity. In trypsin, an aspartate residue (Asp 189) is present at the bottom of the S_1 pocket in place of a serine residue in chymotrypsin. The aspartate residue attracts and stabilizes a positively charged arginine or lysine residue in the substrate. In elastase, two residues at the top of the pocket in chymotrypsin and trypsin are replaced with valine (Val 190 and Val 216). These residues close off the mouth of the pocket so that only small side chains may enter (Figure 9.13).

Other members of the chymotrypsin family include a collection of proteins that take part in blood clotting, to be discussed in Chapter 10. In addition, a wide range of proteases found in bacteria and viruses also belong to this clan. Furthermore, other enzymes that are not homologs of chymotrypsin have been found to contain very similar active sites. As noted in Chapter 7, the presence of very similar active sites in these different protein families is a consequence of convergent evolution. Subtilisin, a protease in bacteria such as *Bacillus amyloliquefaciens*, is a particularly well characterized example. The active site of this enzyme includes both the catalytic triad and the oxyanion hole. However, one of the NH groups that forms the oxyanion hole comes from the side chain of an asparagine residue rather than from the peptide backbone (Figure 9.14). Subtilisin is the founding member of another large family of proteases that includes representatives from Archaea, Eubacteria, and Eukarya.

Yet another example of the catalytic triad has been found in carboxypeptidase II from wheat. The structure of this enzyme is not significantly similar to either chymotrypsin or subtilisin (Figure 9.15). This protein is a member of an intriguing family of homologous proteins that includes esterases such as acetylcholine esterase and certain lipases. These enzymes all make use of histidine-activated nucleophiles, but the nucleophiles may be cysteine rather than serine. Finally, other proteases have been discovered that contain an active-site serine or threonine residue that is activated not by a histidine-aspartate pair but by a primary amino group from the side chain of lysine or by the N-terminal amino group of the polypeptide chain.

Thus, the catalytic triad in proteases has emerged at least three times in the course of evolution. We can conclude that this catalytic strategy must be an especially effective approach to the hydrolysis of peptides and related bonds. TOP

9.1.5. The Catalytic Triad Has Been Dissected by Site-Directed Mutagenesis

The techniques of molecular biology discussed in Chapter 6 have permitted detailed examination of the catalytic triad. In particular, site-directed mutagenesis has been used to test the contribution of individual amino acid residues to the catalytic power of an enzyme. Subtilisin has been extensively studied by this method. Each of the residues within the catalytic triad, consisting of aspartic acid 32, histidine 64, and serine 221, has been individually converted into alanine, and the ability of each mutant enzyme to cleave a model substrate has been examined (Figure 9.16). As expected, the conversion of active-site serine 221 into alanine dramatically reduced catalytic power; the value of k_{cat} fell to less than *one-millionth* of its value for the wild-type enzyme. The value of K_M was essentially unchanged: its increase by no more than a factor of two indicated that substrate binding is not significantly affected. The mutation of histidine 64 to alanine had very similar effects. These observations support the notion that the serine-histidine pair act together to generate a nucleophile of sufficient power to attack the carbonyl group of a peptide bond. The conversion of aspartate 32 into alanine had a smaller effect, although the value of k_{cat} still fell to less than 0.005% of its wild-type value. The simultaneously conversion of all three catalytic triad residues into alanine was no more deleterious than the conversion of serine or histidine alone. Despite the reduction in their catalytic power, the mutated enzymes still hydrolyze peptides a thousand times as rapidly as does buffer at pH 8.6.

Because the oxyanion hole of subtilisin includes a side-chain NH group in addition to backbone NH groups, it is possible to probe the importance of the oxyanion hole for catalysis by site-directed mutagenesis. The mutation of asparagine 155 to glycine reduced the value of k_{cat} to 0.2% of its wild-type value but increased the value of K_M by only a factor of two. These observations demonstrate that the NH group of the asparagine residue plays a significant role in stabilizing the tetrahedral intermediate and the transition state leading to it. TOP

9.1.6. Cysteine, Aspartyl, and Metalloproteases Are Other Major Classes of Peptide-Cleaving Enzymes


Not all proteases utilize strategies based on activated serine residues.

Classes of proteins have been discovered that employ three alternative approaches to peptide-bond hydrolysis (Figure 9.17). These classes are the (1) cysteine proteases, (2) aspartyl proteases, and (3) metalloproteases. In each case, the strategy generates a nucleophile that attacks the peptide carbonyl group (Figure 9.18).

The strategy used by the *cysteine proteases* is most similar to that used by the chymotrypsin family. In these enzymes, a cysteine residue, activated by a histidine residue, plays the role of the nucleophile that attacks the peptide bond (see Figure 9.18), in a manner quite analogous to that of the serine residue in serine proteases. An ideal example of these proteins is papain, an enzyme purified from the fruit of the papaya. Mammalian proteases homologous to papain have been discovered, most notably the cathepsins, proteins having a role in the immune and other systems. The cysteine-based active site arose independently at least twice in the course of evolution; the caspases, enzymes that play a major role in apoptosis (Section 2.4.3), have active sites similar to that of papain, but their overall structures are unrelated.

The second class comprises the *aspartyl proteases*. The central feature of the active sites is a pair of

aspartic acid residues that act together to allow a water molecule to attack the peptide bond. One aspartic acid residue (in its deprotonated form) activates the attacking water molecule by poising it for deprotonation, whereas the other aspartic acid residue (in its protonated form) polarizes the peptide carbonyl, increasing its susceptibility to attack (see Figure 9.18). Members of this class include renin, an enzyme having a role in the regulation of blood pressure, and the digestive enzyme pepsin. These proteins possess approximate twofold symmetry, suggesting that the two halves are evolutionarily related. A likely scenario is that two copies of a gene for the ancestral enzyme fused to form a single gene that encoded a single-chain enzyme. Each copy of the gene would have contributed an aspartate residue to the active site. The human immunodeficiency virus (HIV) and other retroviruses contain an unfused dimeric aspartyl protease that is similar to the fused protein, but the individual chains are not joined to make a single chain (Figure 9.19). This observation is consistent with the idea that the enzyme may have originally existed as separate subunits.

The *metalloproteases* constitute the final major class of peptide-cleaving enzymes. The active site of such a protein contains a bound metal ion, almost always zinc, that activates a water molecule to act as a nucleophile to attack the peptide carbonyl group. The bacterial enzyme thermolysin and the digestive enzyme carboxypeptidase A are classic examples of the zinc proteases. Thermolysin, but not carboxypeptidase A, is a member of a large and diverse family of homologous zinc proteases that includes the matrix metalloproteases, enzymes that catalyze the reactions in tissue remodeling and degradation.

In each of these three classes of enzymes, *the active site includes features that allow for the activation of water or another nucleophile as well as for the polarization of the peptide carbonyl group and subsequent stabilization of a tetrahedral intermediate* (see Figure 9.18). TOP

9.1.7. Protease Inhibitors Are Important Drugs

Compounds that block or modulate the activities of proteases can have dramatic biological effects. Most natural *protease inhibitors* are similar in structure to the peptide substrates of the enzyme that each inhibits (Section 10.5.4). *Several important drugs are protease inhibitors.* For example, captopril, an inhibitor of the metalloprotease angiotensin-converting enzyme (ACE), has been used to regulate blood pressure. Crixivan, an inhibitor of the HIV protease, is used in the treatment of AIDS. This protease cleaves multidomain viral proteins into their active forms; blocking this process completely prevents the virus from being infectious (see Figure 9.19). To prevent unwanted side effects, protease inhibitors used as drugs must be specific for one enzyme without inhibiting other proteins within the body.

Let us examine the interaction of Crixivan with HIV protease in more detail. Crixivan is constructed around an alcohol that mimics the tetrahedral intermediate; other groups are present to bind into the S_2, S_1, S_1', and S_2' recognition sites on the enzyme (Figure 9.20). The results of x-ray crystallographic studies revealed the structure of the enzyme-Crixivan complex, showing that Crixivan adopts a conformation that approximates the twofold symmetry of the enzyme (Figure 9.21). The active site of HIV protease is covered by two apparently flexible flaps that fold down on top of the bound inhibitor. The hydroxyl group of the central alcohol interacts with two aspartate residues of the active site, one in each subunit. In addition, two carbonyl groups of the inhibitor are hydrogen bonded to a water molecule (not shown), which, in turn, is hydrogen bonded to a peptide NH group in each of the flaps. This interaction of the inhibitor with water and the enzyme is not possible with cellular aspartyl proteases such as renin and thus may contribute to the specificity of Crixivan and other inhibitors for HIV protease. TOP

h cb hg e e e fcg c e ch b b c e

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MINI-REVIEW

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An overview on fermentation, downstream processing and properties of microbial alkaline proteases

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Abstract Microbial alkaline proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Of these, strains of *Bacillus* sp. dominate the industrial sector. To develop an efficient enzyme-based process for the industry, prior knowledge of various fermentation parameters, purification strategies and properties of the biocatalyst is of utmost importance. Besides these, the method of measurement of proteolytic potential, the selection of the substrate and the assay protocol depends upon the ultimate industrial application. A large array of assay protocols are available in the literature; however, with the predominance of molecular approaches for the generation of better biocatalysts, the search for newer substrates and assay protocols that can be conducted at micro/nano-scale are becoming important. Fermentation of proteases is regulated by varying the C/N ratio and can be scaled-up using fed-batch, continuous or chemostat approaches by prolonging the stationary phase of the culture. The conventional purification strategy employed, involving e.g., concentration, chromatographic steps, or aqueous two-phase systems, depends on the properties of the protease in question. Alkaline proteases useful for detergent applications are mostly active in the pH range 8–12 and at temperatures between 50 and 70°C, with a few exceptions of extreme pH optima up to pH 13 and activity at temperatures up to 80–90°C. Alkaline proteases mostly have their isoelectric points near to their pH optimum in the range of 8–11. Several industrially important proteases have been subjected to crystallization to extensively study their molecular homology and three-dimensional structures.

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Introduction

Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods, and they produce an abundant, regular supply of the desired product. Furthermore, microbial proteins have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. Despite the long list of protease-producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being 'generally regarded as safe' (GRAS), non-toxic and non-pathogenic. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type (Kumar and Takagi 1999).

Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B amyloliquifaciens*, and *B. mojavensis* (Gupta et al. 2002; Kalisz 1988; Kumar and Takagi 1999; Rao et al. 1998). Another bacterial source known as a potential producer is *Pseudomonas* sp. (Bayoudh et al. 2000; Ogino et al. 1999). Among actinomycetes, strains of *Streptomyces* are the preferred source (Petinate et al. 1999). In fungi, Aspergilli (Chakrabarti et al. 2000; Rajamani and Hilda 1987) is the most exploited group, and *Conidiobolus* sp. (Bhosale et al. 1995), and *Rhizopus* sp. (Banerjee and Bhattacharyya 1993) also produce alkaline protease. Among yeasts, *Candida* sp. has been studied in detail as a potential alkaline protease producer (Poza et al. 2001). Despite this interest in other sources, survey of the literature conclusively shows that *Bacillus* sp. are by far

Table 1 Protease assay methods: an overview. *DNHB* 3,5 Dinitro-hydroxy benzene, *FITC* fluorescein isothiocyanate, *FTC* fluorescein thiocarbamoyl, *ELISA* enzyme-linked immunosorbent assay, *BSA* bovine serum albumin

Qualitative assays		
Assay	Substrates	Reaction involved
Protein agar plate assay	Skim milk, casein, gelatin, BSA, keratin	Enzymatic hydrolysis of substrate creating a zone of clearance in culture
Radial diffusion assay	Skim milk, casein, gelatin, BSA, keratin	Enzymatic hydrolysis of substrate creating a zone of clearance in culture supernatant
Thin layer enzyme assay	Skim milk, casein, gelatin, BSA, keratin, fibrinogen, egg-albumin, mucin, immunoglobulin G	Enzymatic hydrolysis of substrate creating a zone of clearance in broth
Quantitative Assays		
Substrate	Wavelength	Reference
Spectrophotometric assays		
Casein	700 nm 670 nm 500 nm 660 nm 750 nm	McDonald and Chen 1965 Morihara et al. 1967 Chu et al. 1992 Meyers and Ahearn 1977 Ogrydziak et al. 1977
Hammerstein casein	660 nm 275 nm	Barthomeuf et al. 1989 Lee et al. 1998
DNHB casein	366 nm	Gallegos et al. 1996
Immobilized ostazin blue S-2G dyed-casein	620 nm	Safarik 1988a
Thermally modified casein complexed with black drawing ink	400 nm	Safarik 1989
Azocasein	440 nm 340 nm 420 nm 480 nm	Jones et al. 1988 Gibb et al. 1989 Steele et al. 1992 Hanlon and Hodges 1981
Thermally modified azocasein	366–400 nm	Safarik 1987c
Azoalbumin	440 nm	Hanlon et al. 1982
β_L-Crystalline aggregate	405 nm	Cowan and Daniel 1996
Thermally modified gelatin complexed with congo red or nigrosin	490, 570 nm	Safarik 1987a
Chemically modified (formaldehyde/ gluteraldehyde mediated) gelatin complexed with black drawing ink	800–900 nm	Safarik 1989
Tripeptide substrate	400 nm	Gray et al. 1985
Fluorescent oligopeptide energy transfer assay		
Dansylated hexapeptide	310–410 nm	Ng and Auld 1989
ELISA-based protease assay		
Biotinylated BSA	405 nm	Bedouet et al. 1998; Koritsas and Atkinson 1995
Magnet-based protease assay		
Magnet dye stained gelatin	605 nm	Safarikova and Safarik 1999
Fluorescence-based protease assay		
FITC casein, FTC hemoglobin	575 nm with excitation at 490 nm	Twining 1984

the most popular source of commercial alkaline proteases to date, with Novozymes, Denmark being the most established manufacturer worldwide. We have previously discussed molecular approaches for discovering novel proteases and their industrial applications (Gupta et al. 2002). In this review, we discuss some aspects of the various assay protocols presently available for estimating protease activities, and present an overview on fermentation, downstream processing and properties of microbial alkaline proteases.

Protease assays

Proteolytic activity can be measured by either qualitative or quantitative methods. The basic principle underlying

Table 2 Some examples of definitions of protease units from the literature

Definition	Reference
Protease unit: amount of enzyme liberating 1 μmol tyrosine/min under defined conditions	Meyers and Ahearn 1977
Protease unit: one enzyme unit is defined as the amount of enzyme required to increase A_{280} by 0.02	Sharma et al. 1996
Proteolytic unit: amount of enzyme required to solubilize 1 μg casein/h	Griswold et al. 1999
Proteolytic unit: amount of enzyme liberating 1 μg tyrosine/min under defined conditions	Hameed et al. 1999
Proteolytic unit: defined as the amount of enzyme causing an increase in $A_{750\ nm}$ of 0.1 after 1 h	Ogrydziak and Scharf 1982
Proteolytic unit: the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette	McIntyre et al. 2000
Proteolytic unit: the amount of enzyme required to solubilize 1 μg casein/h. Relative proteolytic unit defined as the proteolytic activity U/ml culture supernatant or U/mg cellular protein	Griswold et al. 1999
Proteolytic unit: the amount of enzyme resulting in an increase of 1.0 absorbance unit/ml reaction mixture at 37°C	Kunitz 1947
Proteolytic unit: one unit defined as activity required to increase $A_{340\ nm}$ by 0.001/min	Gibb et al. 1989
Alkaline Delft unit (ADU): an arbitrary unit defined as follows: if 1 ml of a 2% enzyme solution gives a ΔOD of 0.4, then the enzyme preparation has a protease activity of 1,000 ADU/g	Durham et al. 1987
Katal unit: one katal unit of the alkaline protease is defined as the amount of enzyme that yields the color equivalent to 1 mol tyrosine s^{-1} with Folin-Ciocalteau phenol reagent, with casein as substrate at pH 10 and 30°C, according to the enzyme nomenclature recommended by IUB	Tsuchida et al. 1986
Detergent alkaline protease unit (DAPU): the activity liberating 4 nmol tyrosine/min under the assay conditions	Moon and Parulekar 1993
Azocasein unit: $\Delta OD_{420\ nm}$ /h of 1.00 with azocasein as the substrate	Janssen et al. 1994
Azocasein digestion unit: amount of enzyme required to cause an increase of $0.001A_{340}$ U/min under the reaction conditions using azocasein as the substrate	Morita et al. 1998
Hammerstein caseinolytic unit: defined as the amount of enzyme liberating 1 μg tyrosine equivalent/min under specified test conditions	Lee et al. 1998
Anson unit: amount of enzyme that produces absorbance equivalent to 1 mmol tyrosine from casein/10 min under the reaction conditions	Chu et al. 1992
Anson unit: 1 U protease activity defined as the amount of enzyme digesting hemoglobin under standard conditions at an initial rate such that there is liberated/min an amount of split products, not precipitated by TCA, that gives the same color as with the Folin Ciocalteau's phenol reagent with 1 mol equivalent of tyrosine	Anson 1938

both types of method involves measurement of either the products of protein hydrolysis or of residual protein itself. The methods available for detection and assay of proteolytic activity vary in their simplicity, rapidity, range of detection and sensitivity. Table 1 compares various assays used for protease estimation and Table 2 provides a comprehensive list of commonly existing definitions of protease units. The commonly followed assays for the measurement of proteolytic activity are described below.

Qualitative assays

Qualitative assays rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. The most commonly used qualitative assays include protein agar plate assay, radial diffusion and thin-layer enzyme assay.

Protein agar plate assay

Protein agar plate assay is commonly used for the initial screening of proteolytic activity and, depending upon the need and strategy of screening, different protein substrates are selected. The most commonly used protein substrates for selective screening are skim milk (Rajamani and Hilda 1987), casein, gelatin, bovine serum albumin (BSA) (Vermelho et al. 1996) and keratin (Friedrich et al. 1999). The plate assay can also be used to distinguish the type of protease as neutral or alkaline by manipulating the buffer system (Rajamani and Hilda 1987).

Radial (zone) diffusion assay

Radial diffusion or zone diffusion assay (Wikström et al. 1981) is used for semi-quantitative assessment of protease activity. Protease is detected by observing the zone of hydrolysis around small wells cut in agar plates containing the appropriate protein substrate. In another method, the Coomassie prestained substrate agarose gel allows direct assessment of protease activity (Hagen et al. 1997).

Thin-layer enzyme assay

Wikström (1983) developed a thin-layer enzyme assay technique. The main advantage of this assay is that it is a

sensitive, convenient and inexpensive method, which can be used to select specific microbial protease producers in a mixed sample. However, the major disadvantage of this assay is the choice of culture agar medium. It should be more-or-less transparent and should support both optimal growth and enzyme production. Secondly, a high concentration of enzyme substrate in the agar gel is a must.

Quantitative assays

Quantification of protease activity measures the extent of proteolytic potential of the enzyme. The commonly used methods employ natural or synthetic substrates using techniques such as spectrophotometry, fluorimetry, radiometry and enzyme-linked immunosorbent assay-based assays (ELISA).

Spectrophotometric methods

The commonly accepted procedure for estimating the activity of proteases is to determine the quantity of peptides in acid-soluble hydrolyzed product fractions after proteolytic action on a protein substrate (BSA, casein, hammerstein casein, hemoglobin). These peptide residues are estimated either by absorption at 280 nm (direct estimation method) or using conventional Folin's reagent (colorimetric method). Trivedi et al. (1999) used calf lens refolded β_L-crystallin aggregate as substrate for protease detection and assay. This assay can be used for proteases active in the pH range 6–9. Chromogenic or insoluble chromolytic substrates are also used for spectrophotometric determination of proteolytic activity in plants, animals and microbial samples. Both naturally occurring insoluble proteins, e.g., fibrin, elastin, gelatin, keratin, collagen, or soluble proteins rendered insoluble either by cross-linking with bifunctional agents (Safarik 1987a, b; 1989) or entrapment into appropriate polymer matrix (Safarik 1988b), thermally modified substrates (Safarik 1987c, 1988a), or synthesized chromogenic substrates using 3,5-dinitro-salicylic acid (Gallegos et al. 1996) can be used.

Hatakeyama et al. (1992) developed a photometric assay for proteases in which casein, with its amino groups chemically succinylated, was used as the substrate. The extent of hydrolysis of substrate was determined using trinitrobenzene sulfonate (TNBS). The increase in absorbance due to reaction between TNBS and the newly formed amino groups in the substrate was determined using a microtiter plate reader (A_{405nm}). Unlike casein, succinyl casein is easily dissolved at pH values greater than 4 and serves as the substrate of choice for acidic proteases. Colorimetric point assay (using TNBS) is tedious and cannot be used with enzymes that require reducing agents such as dithiothreitol.

A chromogenic tripeptide substrate, benzyloxy carbamoylglycyl-L-prolyl-L-citrulline-*p*-nitroanilide was used to assay a highly alkaline protease, HAP-PB92, from an alkalophilic *Bacillus*, by monitoring release of *p*-nitroaniline by absorbance measurement at 400 nm (Gray et al. 1985). The assay is sensitive and requires only a very short reaction time. The use of soluble chromolytic substrates necessitates the removal of nonhydrolyzed or partially hydrolyzed protein molecules from the assay mixture before absorbance measurement. Insoluble chromolytic substrates, on the other hand, need special care to ensure proper and adequate substrate distribution. In view of the above shortcomings, Wu and Abeles (1995) reported the use of magnetic beads with radiolabelled peptides. This method may replace integration of substrate hydrolysis and separation of proteolytic products. Another, magnet-based, protease assay (Safarikova and Safarik 1999) was used for determining proteolytic activity using dyed magnetic gelatin as an insoluble chromolytic substrate. Such substrates can be used to develop new automated protease assays based on the principle of flow injection analysis.

Fluorescent oligopeptide energy transfer assay

In fluorescent oligopeptide energy transfer assay (Ng and Auld 1989), a fluorescent peptide substrate is designed to explore the protease specificity for the amino acids in the region of the cleavage site (C- and N-terminal). This assay is based on intramolecular quenching of indole fluorescence by an N-terminal dansyl group separated by six amino acid residues. Although this method is sensitive for detection and quantification of specific endoproteases, it is not used much because of the high cost of the assay components.

Enzyme-linked immunosorbent assay

In enzyme-linked immunosorbent assay (ELISA)-based assays (Bedouet et al. 1998), biotinylated-BSA was used as substrate in polystyrene-coated microtiter plates, and the absorbance was recorded at 405 nm using a microtiter ELISA reader. ELISA-based assay methods have the limitation that details of the complete three-dimensional structure of the test enzyme must be known before using an antibody against it. The inhibition ELISA (Clements et al. 1990) is used for detection and quantification of low levels (~0.24 ng/ml) of proteases. It is a very sensitive assay and can be used as a framework for a test system for quantifying proteases in dairy industries. Koritsas and Atkinson (1995) developed a rapid, cheap and sensitive method for determining the proteolytic activity of different classes of endoproteases. The assay is quick, reproducible, inexpensive and suitable for all classes of endopeptidases. In another ELISA-based method, Blair and McDowell (1995) used a double-antibody-sandwich ELISA, which can detect small quantities (up to 4 µg/ml) of protease from *Pseudomonas fragi*.

Fluorescence-based protease assay

Fluorescence-based protease assays are simple, inexpensive and sensitive protease assays using soluble fluorescein isothiocyanate (FITC)-labeled casein (Twining 1984) or FTC-hemoglobin for acid proteases and trypsin (DeLumen and Tappel 1970). The derivative is cleaved by various known proteases, viz. trypsin, chymotrypsin, elastase, subtilisin and thermolysin in a linear, time-dependent, manner. The assay can be used to detect nanogram and sub-nanogram levels of these enzymes with reproducible results.

X-ray based method

This method (Cheung et al. 1991) is based on utilizing gelatin on the surface of an unprocessed Kodak X-Omat AR film as a proteolytic substrate. The assay is convenient, rapid and simple. It can be used for a variety of proteases under a wide pH range of 5 to 8.5. The method is useful as a general laboratory procedure.

Thus, a large array of assay protocols is available for protease estimation. However, in view of their reliability, ease of performance and cost effectiveness, the skim milk agar assay, and casein-based spectrophotometric assays remain the methods of choice for routine laboratory analysis. Additionally, tests with specific substrates can be used for characterizing a protease for specific and selective (keratinolytic, collagenase etc.) applications. Sophisticated methods based on ELISA, inhibition ELISA, fluorescence and radiolabelling can be pursued for very rapid and sensitive quantification of protease activity in microtiter-nanotiter levels and may prove beneficial for characterizing large numbers of clones or enzyme variants. This is often the case while working on modification or modulation of enzyme activity at the molecular level using strategies of directed evolution. Recently, with the growing acceptance of the use of proteases in the detergent, food, and pharmaceutical industries, there is an urgent need to develop highly sensitive and reliable assays capable of screening millions of mutants/variants of proteases to select the desired potential biocatalyst having novel selectivities and kinetic properties.

Regulation of protease biosynthesis

Protease production is an inherent property of all organisms and these enzymes are generally constitutive; however, at times they are partially inducible (Beg et al. 2002a; Kalisz 1988). Proteases are largely produced during stationary phase and thus are generally regulated by carbon and nitrogen stress. Different methods in submerged fermentations have been used to regulate protease synthesis with strategies combining fed-batch, continuous, and chemostat cultures (Gupta et al. 2002; Hameed et al. 1999). Such strategies can achieve high yields of alkaline protease in the fermentation medium over a longer period of incubation during prolonged stationary state. Further, proteases are known to be associated with the onset of stationary phase, which is marked by the transition from vegetative growth to sporulation stage in spore-formers. Therefore, protease production is often related to the sporulation stage in many bacilli, such as *B. subtilis* (O'Hara and Hageman 1990), and *B. licheniformis* (Hanlon and Hodges 1981). On the contrary, a few reports also suggest that sporulation and protease production – although co-occurring – are not related, as spore-deficient strains of *B. licheniformis* were not protease-deficient (Fleming et al. 1995). Our studies on polyamine-mediated regulation of growth, differentiation and protease production in *B. licheniformis* also suggested that the putrescine-to-spermidine ratio also regulates growth and differentiation, but did not affect protease levels. It also established that protease production and sporulation are two independent events in stationary phase (Khan 2000). A similar observation was made in *B. licheniformis* (Bierbaum et al. 1991) by analysis of nucleotide pools (GTP and ATP) in the cells. These results strongly suggested that protease production is under stringent control responsive to amino acid deficiency and is related to the Gppp ratio in the cell. The transitions between different growth phases or different nutritional limitations were easily discerned by the alterations in the nucleotide pool. A marked decrease in the GTP content of the cells (after addition of mycophenolic acid in the exponential phase) increased protease production during stationary phase. Hence, it is conclusively suggested that extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase. However, final protease yield during this phase is also determined by the biomass produced during exponential phase. Therefore, medium manipulation is needed to maximize growth and hence protease yields. Alkaline proteases are generally produced by submerged fermentation and on a commercial scale this is preferred over solid-state fermentation. Optimization of the medium is associated with a large number of physiological and nutritional parameters that effect protease production, viz. pH, temperature, incubation period and agitation, effect of carbon and nitrogen, and divalent cations. Although a large array of factors influences protease production, there is a complex interaction among these parameters that can be studied by following response surface methods. Examples can be referred to in our earlier review (Gupta et al. 2002). A comprehensive account of culture conditions for protease production from various microorganisms is listed in Table 3.

Purification strategies for alkaline proteases

A number of alkaline proteases from different sources have been purified and characterized and a summary of various purification strategies adopted for purification of microbial alkaline proteases is presented in Table 4. There

Table 3 Optimized production conditions for alkaline-protease-producing microorganisms

Microorganism	pH	Temperature (°C)	Agitation (rpm)	Incubation period (h)	Preferred/optimized nitrogen sources	Preferred/optimized carbon sources	Reference
Bacteria							
Alcaligenes faecalis	8	30	200	48	Soybean meal	None[a]	Thangam and Rajkumar 2000
Bacillus sp. IS-3	10.5	37	200	72	Soybean meal	Glucose	Purva et al. 1998
Bacillus sp. JB99	10	55	180	24	KNO_3	Citric acid	Johnvesly and Naik 2001
Bacillus sp. K2	7	37	300–500	60–72	Casein hydrolyzate, gelatine	Glycerol	Hameed et al. 1999
Bacillus sp. P-2	9.5	30	–	24	Peptone, yeast extract	Glucose	Kaur et al. 2001
Bacillus sp. RGR-14	7	37	200	72–96	Soybean meal; peptone	Starch	Oberoi et al. 2001; Puri et al. 2002
Bacillus sp. SSR-1	10	40	150	18	Biopeptone, yeast extract	Beef extract, lactose	Singh et al. 2001a
B. brevis MTCC B0016	10.5	37	200	96	Soybean meal	Lactose	Banerjee et al. 1999
B. licheniformis ATCC 21415	7	30	250–400	48	Soybean, $(NH_4)_2PO_3$	Lactose, glucose	Mabrouk et al. 1999
B. mojavensis	7	50	200–250	24	Casein or casamino acids	Glucose	Beg et al. 2002a
B. pumilis MK6–5	9.6	35	250	60	Cornsteep liquor, tryptone	Glucose, sodium citrate	Kumar 2002
B. sphaericus	n.s.[b]	30	300	n.s.	Biopeptone, yeast extract	Glucose	Singh et al. 2001b
B. subtilis 168	n.s.	36	250	6–8	Nutrient broth; yeast extract	Glucose; yeast extract	Longo et al. 1999
Flavobacterium balustinum P104	7.4	10	150	72	Polypeptone, yeast extract, casein	None[a]	Morita et al. 1998
Serratia marcescens ATCC 25419	n.s.	30; 36	250	24; 16–18	Yeast extract, tryptone, asparagine, NH_4Cl	Whey; sucrose	Romero et al. 2001; Longo et al. 1999
Fungi							
Conidiobolus coronatus (NCL 86.8.20)	7–7.5	28	220	48	Ammonium nitrate, tryptone, casein	Sucrose	Bhosale et al. 1995
Ophiostoma piceae	n.s.	23	250	9 days	Soydrink from soybean meal	Starch	Abraham and Breuil 1996
Tritirachium album	5.9	28	200	24–120	Peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$	Glucose	Fortelius and Markkanen 2000
Actinomycetes							
Streptomyces sp. NCIB 10070	7	n.s.	n.s.	24	Rapemeal	None[a]	Yeoman and Edwards 1997
Thermoactinomyces sp. E79	7.2	50	250	16	Soytone	Starch	Lee et al. 1996

[a] No carbon source was present in the medium and the major organic nitrogen source supplied the required carbon
[b] Not specified

are no set rules for the purification of proteases. After separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultrafiltration (Kang et al. 1999; Smacchi et al. 1999), salting out by solid ammonium sulfate (Kumar 2002; Hutadilok-Towatana et al. 1999), or solvent extraction methods using acetone (Kumar et al. 1999; Thangam and Rajkumar 2002) and ethanol (El-Shanshoury et al. 1995). In addition, other methods, such as use of PEG-35,000 (Larcher et al. 1996), activated charcoal (Aikat et al. 2001), temperature-sensitive hydrogel (Han et al. 1995), heat treatment of enzyme (Rahman et al. 1994) and lyophilization (Manonmani and Joseph 1993) are also used for concentration of alkaline proteases.

To further purify the enzyme, a combination of one or more techniques is applied, viz. affinity chromatography (AC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and gel filtration chromatography. Other methods of choice, including aqueous two-phase systems (Sinha et al. 1996), dye ligand chromatography (Cowan and Daniel 1996), and foam fractionation (Banerjee et al. 1993), have also been employed on a small scale and still await commercial exploitation.

Affinity chromatography

The most common affinity adsorbents used for alkaline proteases are hydroxyapatite (Kobayashi et al. 1996), immobilized *N*-benzoyloxycarbonyl phenylalanine agarose (Larcher et al. 1996), immobilized casein glutamic acid (Manonmani and Joseph 1993), aprotinin-agarose (Petinate et al. 1999), and casein-agarose (Hutadilok-Towatana et al. 1999). Although AC is one of the most successful purification techniques, a major limitation is the high cost of enzyme supports and the labile nature of the affinity ligands, which lowers their use at process scale.

Ion exchange chromatography

Generally, alkaline proteases are positively charged and they are not bound to anion exchangers (Kumar and Takagi 1999). Therefore, cation exchangers are the methods of choice. The matrices for IEC contain ionizable functional groups such as diethyl amino ethyl (DEAE) and carboxy methyl (CM), which get associated with the charged protein molecules, thereby adsorbing the protein to the matrices. The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution.

Hydrophobic interaction chromatography

HIC exploits the variability of external hydrophobic amino acid residues on different proteins, leading to protein interaction by virtue of the fact that in aqueous solvents, hydrophobic patches on proteins preferentially seek out other hydrophobic surfaces. These hydrophobic interactions are strengthened by high salt concentrations and higher temperatures, and are weakened by the presence of detergents or miscible organic solvents. The extent of binding of a hydrophobic protein depends on the type and density of substitution of the matrix, as well as on the nature of buffer conditions. Hydrophobic interactions are much more variable in behavior than ion exchangers and, because of this, resolution is generally poorer than IEC. HIC has been extensively used in FPLC in various columns, such as Mono-Q HR 5/5 (Rattray et al. 1995; Smacchi et al. 1999), Econo-pac Q (Yeoman and Edwards 1997), and Mono S 5/10 (Yum et al. 1994). The most commonly used hydrophobic adsorbents are octyl- (C_8-) and phenyl-substituted matrices.

Affinity precipitation

Affinity precipitation is a function of a soluble macromolecule (ligand polymer and macroligand) that has two functions: (1) it contains an affinity ligand (preferably more, polyvalent macromolecule), and (2) it can be precipitated in many ways, i.e., by change in pH, temperature or ionic strength. With this technique, the ligand polymer is added to the enzyme solution under conditions favoring binding of the protein of interest. The ligand polymer is then precipitated, and the supernatant is removed. The protein of interest is then eluted from the polymer under suitable conditions, and the polymer can be recycled. An alkaline protease (Maxatase from *B. licheniformis*), used as a washing powder additive, has been purified by affinity precipitation (Pecs et al. 1991).

In addition to the above chromatographic techniques, gel filtration is used for rapid separation of macromolecules based on size. Recently, many new agarose-based and more rigid and cross-linked gels, such as Sephacryl, Superose, Superdex and Toyopearl are also being used for purification purposes. They are generally used either in the early-to-middle stage of purification (Chakrabarti et al. 2000) or in the final stages of purification (El-Shanshoury et al. 1995; Lee et al. 1996). Major disadvantages of this method are the lower capacity for loading proteins and that the desired protein gets too diluted.

Properties of alkaline proteases

Alkaline proteases from several microorganisms have been studied extensively and, based on their properties, used in various industries. The important properties are summarized in Table 5. However, a brief account of individual properties is presented in the following section.

Table 4 Combination of purification techniques applied to purification of various alkaline proteases. *DEAE* Diethyl amino ethyl, *CM* carboxy methyl

Microorganism	Concentration method	Column matrices	Reference
Bacteria			
Alcaligenes faecalis	Acetone	DEAE-cellulose, Sephadex G-100	Thangam and Rajkumar 2002
Arthrobacter nicotianae 9458	Ultrafiltration	DEAE-Sephacyl, Sephacryl 200, Phenyl Sepharose, FPLC Mono Q HR 5/5	Smacchi et al. 1999
Bacillus sp. PS719	$(NH_4)_2SO_4$	DEAE-cellulose, α-casein agarose	Hutadilok-Towatana et al. 1999
Bacillus sp. NCDC 180	Acetone, $(NH_4)_2SO_4$	DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200	Kumar et al. 1999
Bacillus sp. SSR1	$(NH_4)_2SO_4$	DEAE-Sephadex A-50, Sepharose 6B	Singh et al. 2001a
B. pumilis MK6–5	$(NH_4)_2SO_4$	DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200	Kumar 2002
B. sphaericus MTCC B-0014	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl agarose, Q-Sepharose	Singh et al. 2001b
Oligotropha carboxydovorans DSM 1227	Ultrafiltration	Sephadex G-75, CM-cellulose	Kang et al. 1999
Pimelobacter sp. Z-483	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-50F	Oyama et al. 1997
Pseudomonas aeruginosa MN1	$(NH_4)_2SO_4$	Sephadex G-100, DEAE-cellulose	Bayoudh et al. 2000
P. aeruginosa PST-01	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M	Ogino et al. 1999
Serratia marcescens ATCC 25419	$(NH_4)_2SO_4$	Q-Sepharose, Sephacryl S-200	Romero et al. 2001
Fungi			
Aspergillus terreus (IJIRA 6.2)	Acetone, $(NH_4)_2SO_4$	DEAE-Sephadex A25, SDS-PAGE, electroelution	Chakrabarti et al. 2000
Scedosporium apiospermum	$(NH_4)_2SO_4$, PEG 35,000	Sephadex G-75, immobilized phenylalanine-agarose	Larcher et al. 1996
Actinomycetes			
Oerskovia xanthineolyrica TK-1	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl-Sepharose CL 4B, DEAE-Sephacel	Saeki et al. 1994
Streptomyces cyaneus	–	Aprotinin-agarose	Petinate et al. 1999
Streptomyces thermovulgaris	Ultrafiltration	FPLC Enono-pac Q, Seperose 12	Yeoman and Edwards 1997
Thermoactinomyces sp. E79	$(NH_4)_2SO_4$,	DEAE-Sepharose CL-6B, Butyl-toyopearl 650 M	Lee et al. 1996
Moulds			
Aureobasidium pullulans	Ultrafiltration	Sephadex G-75	Donaghy and McKay 1993
Candida caseinolytica	–	Sephacryl S-200, DEAE-Biogel	Poza et al. 2001

pH and temperature kinetics

In general, all currently used detergent-compatible proteases are alkaline and thermostable in nature with a high pH optimum – the pH of laundry detergents is generally in the range of 8 to 12 – and have varying thermostabilities at laundry temperatures (50–70°C). Therefore, most of the commercially available subtilisin-type proteases are also active in the pH and temperature ranges 8–12 and 50–70°C, respectively (Table 5). In addition, a recent trend in the detergent industry is a requirement for alkaline protease active at low washing temperatures; for example, Kannase – marketed by Novozymes – is active even at temperatures as low as 10–20°C.

Effect of stabilizers/additives and metal ions

Some of the major commercial uses of alkaline proteases necessitate high temperatures, thus improving the thermal stability of the enzyme is distinctly advantageous. Thermostability can be enhanced either by adding certain stabilizers (PEG, polyhydric alcohols, starch) to the reaction mixture or by manipulating the tertiary structure of enzyme by protein engineering. A thermostabilization effect of up to a 2-fold increase in the half-life of *Cucurbita ficifolia* protease at 65°C has been reported by using polyhydric alcohols, PEG and casein (Gonzalez et al. 1992). The ion Ca^{2+} is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures (Kumar 2002; Lee et al. 1996). Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} are also used for stabilizing proteases (Johnvesly and Naik 2001; Rattray et al. 1995). These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures.

Substrate specificity

Alkaline proteases have broad substrate specificity and are active against a number of synthetic substrates and natural proteins. However, the literature conclusively suggests that they are more active against casein than against azocasein, hemoglobin or BSA (Table 5). Moreover, there are specific types of alkaline proteases, viz. collagenase, elastase, keratinase (Friedrich et al. 1999) and insect cuticle-degrading protease (Urtz and Rice 2000), which are active against specific protein substrates (such as collagen, elastin, keratin, cuticle). Alkaline proteases are also specific against aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine or leucine at the carboxylic side of the cleavage site.

Kinetic parameters

To develop an enzyme-based process, prior information about kinetic parameters of the enzyme in question is of utmost importance. To be precise, kinetic properties like V_{max}, K_m, K_{cat}, and E_a are important, being not only enzyme-specific, but also substrate- and environment-specific, and knowledge of these is essential for designing enzyme reactors or quantifying the applications of the enzyme under different conditions. Various complex, viz., casein, azocasein etc., and synthetic substrates, viz., para-nitroanilides esters, are used for determining kinetic parameters for alkaline proteases. The synthetic substrates are much more popular than complex substrates for defining K_m and V_{max} as they are convenient (Kumar 2002; Larcher et al. 1996). For an alkaline protease from *B. mojavensis*, the K_m for casein decreased with corresponding increase in V_{max}, as the reaction temperature was raised from 45 to 60°C (Beg et al. 2002b). In contrast, the K_m and V_{max} for an alkaline protease from *Rhizopus oryzae* increased with an increase in temperature from 37°C to 70°C (Banerjee and Bhattacharyya 1993).

Crystallization of serine proteases

Knowledge of the tertiary structure of an enzyme is essential to the understanding of its catalytic function. Of particular importance is information about possible changes in enzyme structure due to binding of substrate, products, or effector molecules. Clearly, the spatial distribution of groups at the active site and their geometrical disposition has to be known as accurately as possible. Therefore, in order to deduce the factors dominant in any reaction system, a knowledge of refined protein structure at high resolution is of paramount importance. Developments in X-ray crystallography have revolutionized our understanding of the structure-function relationship of proteins. These complex molecules provide the machinery to drive and sustain the living cell and ensure that the vital genetic information contained in the DNA is passed on to future generations. Serine proteases of the subtilisin family have been extensively studied both to provide insight into the mechanism and specificity of enzyme catalysis and because of their considerable industrial importance. Many mutually homologous serine proteases has been subjected to crystallization and 3-dimensional structures of proteases from subtilisin BPN' and subtilisin Novo from *B. amyloliquefaciens* (Bott et al. 1988; Drenth et al. 1972; Erwin et al. 1990; Wright et al. 1969), subtilisin Carlsberg from *B. licheniformis* (Bode et al. 1987; Niedhart and Petsko 1988), subtilisin (Kuhn et al. 1998) and Savinase (Betzel et al. 1988a, 1992) from *B. lentus*, opticlean from *B. alcalophilus* (Sobek et al. 1990), proteinase K from *Tritirachium album* Limber (Betzel et al. 1988b; Singh et al. 2001c), thermitase from *Thermoactinomyces vulgaris* (Teplyakov et al. 1990), and serine protease from *Penicillium cyclopium* (Day et al. 1986; Koszelak et al. 1997) have been determined by X-ray

Table 5 Properties of some alkaline proteases from different microbial sources

Microorganism	pH optima	Temperature optima (°C)	Substrate specificity	MW (kDa)	pI	Other properties	Reference
Bacteria							
Alcaligenes faecalis	9	55	Casein, BSA, gelatin, azocoll, azocasein	67	n.s.[a]	–	Thangam and Rajkumar 2002
Arthrobacter nicotianae 9458	9; 9.5	55–60; 37	α_{S1}- and β-casein	55; 70–72	n.s.	–	Smacchi et al. 1999
Bacillus sp. JB99	11	70	Casein	29	n.s.	Metal ions enhance thermostability	Johnvesly and Naik 2001
Bacillus sp. NG-27	9.2	40	Casein	n.s.	n.s.	Half life of 55 min at 90°C	Sumandeep et al. 1999
Bacillus sp. KSM-KP43	11	70	Casein	n.s.	n.s.	Oxidation-resistant	Saeki et al. 2002
Bacillus sp. NCDC-180	11; 12	50; 55	Casein, synthetic *p*-nitroanilides	28; 29	n.s.	Stable up to pH 13	Kumar et al. 1999
Bacillus sp. PS179	9	75	Azocasein	42	4.8	Ca^{2+} enhances thermostability	Hutadilok-Towatana et al. 1999
Bacillus sp. SSR1	10	40	Azocasein	29	n.s.	Ca^{2+} enhances thermostability	Singh et al. 2001a
B. brevis MTCC B0016	10.5	37	Azocasein	n.s.	n.s.	Detergent compatible	Banerjee et al. 1999
B. mojavensis	10.5	60	Casein	30	n.s.	Bleach- and SDS-stable, detergent compatible	Beg et al. 2002b; Gupta et al. 1999
B. pumilis MK6–5	11.5	50–55	*p*-Nitroanilides	28	n.s.	Ca^{2+}-independent	Kumar 2002
Oligotropha carboxydovorans DSM 1227	9	60; 50	Casein, azocasein, azocoll, carbon monooxide dehydrogenase	23	n.s.	–	Kang et al. 1999
Pimelobacter sp. Z-483	9	50	Casein	23	n.s.	EDTA-resistant	Oyama et al. 1997
Pseudomonas aeruginosa PST-01	8.5	55	Casein	38	n.s.	Organic solvent stable	Ogino et al. 1999
Serratia marcescens ATCC 25419	9.5	48	Azocasein	66.5	n.s.	–	Romero et al. 2001
Fungi							
A. terreus (IJIRA 6.2)	8.5	37	Na-caseinate, synthetic substrates (*p*-nitroanilidines)	37	n.s.	–	Chakrabarti et al. 2000
Beauveria bassiana	7.5–9.5	25	Azocoll, elastase	31.5	7.5	Elastase and cuticle degradation activity	Urtz and Rice 2000
Actinomycetes							
Oerskovia xanthineolytica TK-1	9.5–11	50	Synthetic esters	20	n.s.	Yeast-lytic activity	Saeki et al. 1994
Streptomyces cyaneus	9	25	α-*N*-*p*-Tosyl-L-arginine methyl ester	30, 120	n.s.	–	Petinate et al. 1999
Thermoactinomyces sp. E79	11	85	Casein	31		Calcium enhanced thermostability, broad pH stability (5–12)	Lee et al. 1996
Moulds							
Aureobasidium pullulans	9.5–10.5	41	Azocoll, α-casein	27	n.s.	–	Donaghy and McKay 1993
Candida caseinolytica	4.5–11	37	Casein	30	4.7	–	Poza et al. 2001

[a] Not specified

Table 6 Crystallization conditions for subtilisins and other alkaline proteases. *PMSF* Phenyl methyl sulfonyl fluoride

Source and type	Crystallization conditions	Reference
Bacillus amyloliquefaciens (subtilisin BPN')	0.5 M sodium acetate buffer (pH 5.8–6), 1.3 M $(NH_4)_2SO_4$	Wright et al. 1969
Bacillus amyloliquefaciens (subtilisin)	27% saturated $(NH_4)_2SO_4$, 0.5 M sodium acetate (pH 6), 10 mM $CaCl_2$ and PMSF	Bott et al. 1988
B. alcalophilus PB92 (serine protease)	30% PEG-6000 as precipitant in 0.1 M potassium phosphate buffer (pH 6.0)	Laan et al. 1992
B. alcalophilus (opticlean)	1–1.2 M $(NH_4)_2SO_4$ in 50 mM ammonium acetate buffer (pH 5.6–6)	Sobek et al. 1990
B. lentus (Savinase)	12–15% PEG-4000 or 22% $(NH_4)_2SO_4$ as precipitant, 2 mM $CaCl_2$, 1 mM PMSF, 50 mM citrate buffer (pH 6)	Betzel et al. 1988a, 1992
B. licheniformis (Subtilisin Carlsberg)	0.5 M citrate buffer (pH 8) or 1 M phosphate buffer (pH 5.5–6.5) in 1 M $(NH_4)_2SO_4$ or 6–10% PEG-6000 at 20°C	Bode et al. 1987; Neidhart and Petsko 1988
Bacillus sp. (Subtilisin Novo)	10–15% PEG-4000 in 0.1 M potassium phosphate buffer (pH 6)	Drenth et al. 1972; Heinz et al. 1991
B. amyloliquefaciens (subtilisin BPN' gene expressed in *B. subtilis* BG84)	20 mM MES buffer (pH 7); and 35–40% saturated $(NH_4)_2SO_4$, 0.5% PEG 8000, 1% -octylglucoside and 2% 2-methyl-2,4-pentadiol in 50 mM MES buffer (pH 7) in reservoir	Erwin et al. 1990
B. lentus (subtilisin)	(1) 0.5 M sodium acetate buffer (pH 5.9), 10 mM $CaCl_2$, PMSF, 30–33% saturated $(NH_4)_2SO_4$ (2) Succinyl-alanyl-prolyl-phenylalanine-*p*-nitro-anilide, sodium acetate buffer and 22–24% saturated $(NH_4)_2SO_4$	Kuhn et al. 1998
Alkalophilic *Bacillus* sp. NKS-21 (serine protease)	10 mM Atkins and Pantin's buffer (pH 8) containing 2 mM Ca-acetate and 0.2 M NaCl	Tsuchida et al. 1986
Penicillium cyclopium (serine protease)	10–24% PEG-4000 or 15% PEG-3350 as precipitant	Day et al. 1986; Koszelak et al. 1997
Streptomyces fradiae ATCC 14554	10 mM Tris-HCl buffer (pH 7.5) in 1.3 M sodium phosphate (pH 4.2) and 5% dioxane as precipitant in reservoir	Kitadokoro et al. 1994
S. griseus (serine protease *A*)	1.3 M NaH_2PO_4 (pH 4.1) as precipitating salt	Sielecki et al. 1979
Thermoactinomyces vulgaris (Thermitase)	0.1 M sodium phosphate buffer (pH 5.6) with addition of 2–4% 2-methyl-2,4-pentadiol and 20–25% $(NH_4)_2SO_4$	Teplyakov et al. 1990
Tritirachium album Limber (Proteinase K)	50 mM Tris-HCl buffer, 10 mM $CaCl_2$, 1 M $NaNO_2$, 0.02% NaN_3 at pH 6.5	Betzel et al. 1988b; Singh et al. 2001c

diffraction. The crystal structures have been widely studied in detail to provide insight into the mechanism and specificity of enzyme catalysis. The large base of subtilisins and their industrial importance makes them attractive model systems for crystallographic studies and protein engineering, e.g., the role of Ca^{2+} ions in structure-function of subtilisin was extensively studied by X-ray structure analysis of subtilisin BPN' (Pantoliano et al. 1988). Ca^{2+} ions were also located in 3-D structures of subtilisin Carlsberg (Bode et al. 1987), proteinase K (Betzel et al. 1988b) and thermitase (Teplyakov et al. 1990). Although the overall tertiary fold of subtilisin is conserved in proteinase K and thermitase, they have distinctly different Ca^{2+}-binding sites, which may be related to their thermostability. A brief overview of various conditions for crystallization of subtilisins and alkaline proteases is listed in Table 6.

Conclusions

Alkaline proteases constitute a very large and complex group of enzymes, with both nutritional and regulatory roles in nature. They are produced by myriad microorganisms but the most exploited industrial producer belongs to the genus *Bacillus*. Production and downstream processing to obtain purified enzyme sample has been studied extensively, both at laboratory and industrial scale. Moreover, various types of alkaline proteases have been characterized and their potential industrial applications have been explored. The major applications of these enzymes are in detergent formulation, the food industry, leather processing, chemical synthesis and waste management. Given the commercial success of this enzyme, researchers have also started looking into the possibility of developing robust enzymes with desired properties for industrial processes by protein engineering and genetic manipulation. Hence, although microbial alkaline proteases already play an important role in industry, their potential is much greater and their application in future processes is likely to increase.

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Alkaliphiles: Some Applications of Their Products for Biotechnology

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DEFINITION OF ALKALIPHILES

There are no precise definitions of what characterizes an alkaliphilic or alkalitolerant organism. Several microorganisms exhibit more than one pH optimum for growth depending on the growth conditions, particularly nutrients, metal ions, and temperature. In this review, therefore, the term "alkaliphile" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5 (Fig. 1).

HISTORY OF ALKALIPHILES

The discovery of alkaliphiles was fairly recent. Only 16 scientific papers on the topic could be found when I started experiments on alkaliphilic bacteria in 1968. The use of alkaliphilic microorganisms has a long history in Japan, since from ancient times indigo has been naturally reduced under alkaline conditions in the presence of sodium carbonate. Indigo from indigo leaves is reduced by particular bacteria that grow under these highly alkaline conditions in a traditional process called indigo fermentation. The most important factor in this process is the control of the pH value. Formerly, indigo reduction was controlled only by the skill of the craftsman. Microbiological studies of the process, however, were not conducted until the rediscovery of these alkaliphiles by me (69). Alkaliphiles remained little more than interesting biological curiosities, and at that time no further industrial application was attempted or even contemplated.

Since then, my colleagues and I have isolated a large number of alkaliphilic microorganisms and purified many alkaline enzymes. The first paper concerning an alkaline protease was published in 1971 (67). Over the past two decades, our studies have focused on the enzymology, physiology, ecology, taxonomy, molecular biology, and genetics of these isolates to establish a new microbiology of alkaliphilic microorganisms. Industrial applications of these microorganisms have also been investigated extensively, and some enzymes, such as alkaline proteases, alkaline amylases, and alkaline cellulases, have been put to use on an industrial scale. Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore their genes are a potentially valuable source of information waiting to be explored and exploited by the biotechnologists. Genes responsible for the alkaliphily of *Bacillus halodurans* C-125 and *Bacillus firmus* OF4 have been analyzed (49, 181). Recently, the complete genome of *B. halodurans* C-125 has been sequenced to obtain more information on industrial applications and on the molecular basis of alkaliphily. However, in this review, structural, physiological, and genetic aspects of alkaliphiles are not discussed; instead, the review focuses on studies of extracellular alkaline enzymes.

DISTRIBUTION AND ISOLATION OF ALKALIPHILES

Alkaliphiles consist of two main physiological groups of microorganisms; alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH (>pH 9) and high salinity (up to 33% [wt/vol] NaCl). Alkaliphiles have been isolated mainly from neutral environments, sometimes even from acidic soil samples and feces. Haloalkaliphiles have been mainly found in extremely alkaline saline environments, such as the Rift Valley lakes of East Africa and the western soda lakes of the United States.

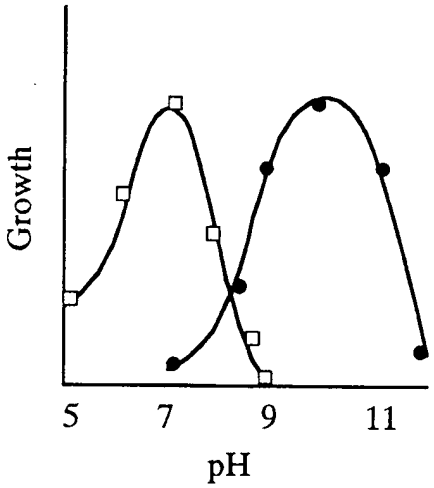

FIG. 1. pH dependency of alkaliphilic microorganisms. The typical pH dependency of the growth of neutrophilic and alkaliphilic bacteria is shown by open squares and solid circles, respectively.

Alkaliphiles

Aerobic alkaliphiles. Alkaliphilic microorganisms coexist with neutrophilic microorganisms, as well as occupying specific extreme environments in nature. Figure 2 illustrates the relationship between the occurrence of alkaliphilic microorganisms and the pH of the sample origin. To isolate alkaliphiles, alkaline media must be used. Sodium carbonate is generally used to adjust the pH to around 10, because alkaliphiles usually require at least some sodium ions. Table 1 shows the makeup of an alkaline medium suitable for their isolation. The frequency of alkaliphilic microorganisms in neutral "ordinary"

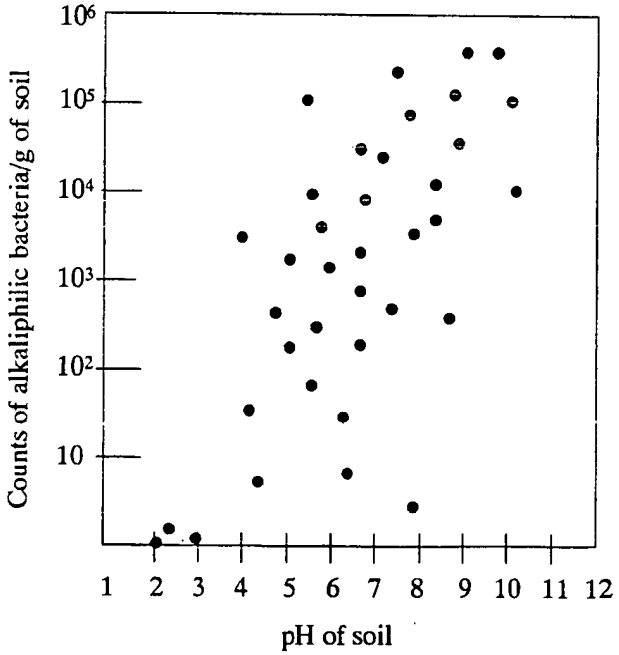

FIG. 2. Distribution of alkaliphilic microorganisms in environments at various pHs.

TABLE 1. Basal media for alkaliphilic microorganisms

Ingredient	Amt (g/liter) in:	
	Horikoshi-I	Horikoshi-II
Glucose	10	0
Soluble starch	0	10
Yeast extract	5	5
Polypeptone	5	5
K_2HPO_4	1	1
$Mg_2SO_4 \cdot 7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20

soil samples is 10^2 to 10^5/g of soil, which corresponds to 1/10 to 1/100 of the population of the neutrophilic microorganisms (65). Recent studies show that alkaliphilic bacteria have also been found in deep-sea sediments collected from depths up to the 10,898 m of the Mariana Trench (180). Many different kinds of alkaliphilic microorganisms, including bacteria belonging to the genera *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Streptomyces* and eukaryotes such as yeasts and filamentous fungi, have been isolated from a variety of environments (30, 50, 65).

Anaerobic alkaliphiles. The first brief report on anaerobic alkaliphiles was communicated by Niimura et al. (144), although no taxonomic details were reported. Subsequently, many anaerobic spore-forming alkaliphiles were isolated by conventional procedures, and a few applications have been studied. Podkovyrov and Zeikus recorded the isolation and purification of a cyclomaltodextrin glucanotransferase from *Clostridium thermohydrosulfuricum* 39E (155). Wiegel and colleagues (32, 117) have isolated and characterized a range of thermophilic anaerobic alkaliphiles. Nine moderately alkalitotolerant thermophilic bacteria with similar properties were isolated from water and soil samples obtained from Yellowstone National Park. Strain JW/YL-138T and eight similar strains represent a new genus and species, *Anaerobranca horikoshii*. At 60°C the pH range for growth is 6.9 to 10.3 and the optimum pH is 8.5. Cook et al. reported that *Clostridium paradoxum*, a novel alkali-thermophile, could maintain pH homeostasis inside the cell and thrive well in culture media in the pH range between 7.6 and 9.8 (23).

Kodama and Koyama (113) studied unique characteristics of anaerobic alkaliphiles belonging to *Amphibacillus xylanus*. The transport of leucine and glucose was remarkably stimulated by NH_4^+. An ($NH_4^+ + Na^+$)-activated ATPase of *A. xylanus* was also reported by Takeuchi et al. (183). Primary amines also activated the enzyme, but secondary and tertiary amines were ineffective (183).

Until recently, few microbiologists thought that alkaliphiles thrived in hydrothermal areas. Stetter's group (184) isolated *Thermococcus alcaliphilus* sp. nov., a new hyperthermophilic archaeon growing on polysulfide at alkaline pH and at temperatures between 56 and 90°C, from a shallow marine hydrothermal system at Vulcano Island, Italy. The pH range for growth was 6.5 to 10.5, with an optimum around 9.0. Polysulfide and elemental sulfur were reduced to H_2S. Then, from a shallow beach (depth 1.0 m) situated at the base of the reef close to Porto di Levante at Vulcano Island, Italy, Dirmeier et al. isolated a new hyperthermophilic member of the archaea (29). The cells are coccoid and have up to five flagella. They grow between 56 and 93°C (optimum, 85°C) and at pH 5.0 to 9.5 (optimum, 9.0). The organism is strictly anaerobic and grows heterotrophically on defined amino acids and complex organic substrates such as Casamino Acids, yeast extract, peptone, meat extract, tryptone, and casein. DNA-DNA hybridization and 16S rRNA partial sequences indicated that the new isolate belongs to the genus *Thermococcus* and represents a new species, *Thermococcus acidaminovorans*. An outstanding review on anaerobic alkalithermophiles has recently been published by Wiegel (198).

Haloalkaliphiles

The most remarkable examples of naturally occurring alkaline environments are soda deserts and soda lakes. Extremely alkaline lakes, for example, Lake Magadi in Kenya and the Wadi Natrun in Egypt, are probably the most stable highly alkaline environments on Earth, with a consistent pH of 10.5 to 12.0 depending on the site. Many organisms isolated from alkaline and highly saline environments such as soda lakes also require high salinity, which is achieved by adding NaCl to the isolation medium. In particular, hypersaline soda lakes are populated by alkaliphilic representatives of halophilic archaea. These red-pigmented microbes reach numbers of 10^7 to 10^8/ml in soda lake brines. A novel haloalkaliphilic archaeon was isolated from Lake Magadi (125). Cells of this organism contain large gas vacuoles in the stationary phase of growth, and colonies produced by these archaea are bright pink. The G+C content of the DNA is 62.7 mol%. The name *Natronobacterium vacuolata* sp. nov. is proposed. The type strain is designated NCIMB 13189. Lodwick et al. (121) determined the nucleotide sequence of the 23S and 5S rRNA genes from the haloalkaliphilic arachaeon *Natronobacterium magadii*. A phylogenetic tree for the halobacteria was produced by alignment of the 23S rRNA sequence with those of other archaea.

Subsequently, Kamekura et al. (81) studied the diversity of alkaliphilic halobacteria on the basis of phylogenetic tree reconstructions, signature bases specific for individual genera, and sequences of spacer regions between 16S and 23S rRNA genes. They proposed the following changes: *Natronobacterium pharaonis* to be transferred to *Natronomonas* gen. nov. as *Natronomonas pharaonis* gen. nov. comb. nov.; *Natronobacterium vacaolatum* to be transferred to the genus *Halorubrum* as *Halorubrum vacuolatum* comb. nov.; and *Natronobacterium magadii* to be transferred to the genus *Natrialba* as *Natrialba magadii*.

Recently, Xu et al. (200) isolated two haloalkaliphilic archaea from a soda lake in Tibet. The two strains were gram negative, pleomorphic, flat, nonmotile, and strictly aerobic. Growth required at least 12% NaCl and occurred between pH 8.0 and 11 with an optimum at pH 9.0 to 9.5. On 16S rRNA phylogenetic trees, the two strains formed a monophyletic cluster. They differed from their closest neighbors, *Halobacterium trapanicum* and *Natrialba asiatica*, in polar lipid composition, as well as physiological and phenotypic characteristics. DNA-DNA hybridization indicated that the two strains belonged to different species of the same genus. The results indicated that the strains should be classified in a new genus, *Natronorubrum* gen. nov.

Recently, Kevbrin et al. (89) isolated an alkaliphilic, obligately anaerobic, fermentative, asporogenous bacterium with a gram-positive cell wall structure from soda deposits in Lake Magadi, Kenya. 16S rDNA sequence analysis of this bacterium showed that it belongs phylogenetically to cluster XI of the low-G+C gram-positive bacteria. On the basis of its distinct phylogenetic position and unique physiological properties, they proposed a new genus and new species, *Tindallia magadii*, for this strain.

A novel osmolyte, 2-sulfotrehalose, was discovered in sev-

eral *Natronobacterium* species of haloalkaliphilic archaea (26). The concentration of this novel disaccharide increased with increasing concentrations of external NaCl, behavior consistent with its identity as an osmolyte. Other common osmolytes (glycine, betaine, glutamate, and proline) were not accumulated or used for osmotic balance in place of the sulfotrehalose by these halophilic archaea. The ecology, physiology, and taxonomy of haloalkaliphiles have been recently reviewed by Jones et al. (80).

Methanogens

Boone et al. (16) had originally isolated halophilic methanogens from various neutral salinas and natural hypersaline environments. These strains grew fastest at temperatures near 40°C and, in medium containing 0.5 to 2.5 M NaCl, at pH values near 7. Zhilina and Zavarzin (206) then described bacterial communities in alkaline lakes, and in particular the diversity of anaerobic bacteria developing at pH 10 was discussed. A new obligate alkaliphilic, methylotrophic methanogen was isolated from Lake Magadi. According to its phenotypic and genotypic properties, the isolate belonged to *Methanosalsus* (*Methanohalophilus*) *zhilinaeae* (88). It exhibited 91% homology to the type strain of this formerly monotypic species. The strain did not require Cl^- but was obligately dependent on Na^+ and HCO_3^-. It was an obligate alkaliphile and grew within a pH range of 8 to 10 (92).

Cyanobacteria

Gerasimenko et al. (48) reported the discovery a wide diversity of alkaliphilic cyanobacteria (16 genera and 34 species were found). So far, no industrial application has been reported (11, 31, 128, 156, 199). Buck and Smith (19) reported an Na^+/H^+ electrogenic antiporter in alkaliphilic *Synechocystis* sp. Singh (165) partially purified a urease from an alkaliphilic diazotrophic cyanobacterium, *Nostoc calcicola*. The purified enzyme showed optimum activity at pH 7.5 and 40°C. The enzyme was found to be sensitive to metal cations, particularly Hg^{2+}, Ag^+, and Cu^{2+}. 4-Hydroxymercuribenzoate (a mercapto-group inhibitor) and acetohydroxamic acid (a chelating agent of nickel) inhibited the enzyme activity completely. These results suggest the involvement of an SH group and Ni^{2+} in the activity of urease from *N. calcicola*.

Other Alkaliphiles

Sorokin et al. have extensively studied sulfur-oxidizing alkaliphiles. The strains oxidize sulfide, elemental sulfur, and thiosulfate to tetrathionate. Recently, sulfide dehydrogenase was isolated and purified from these alkaliphilic chemolithoautotrophic sulfur-oxidizing bacteria (166–169).

Zhilina et al. isolated and characterized *Desulfonatronovibrio hydyogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium, from Lake Magadi. Strain Z-7935(T) is an obligatory sodium-dependent alkaliphile, which grows in sodium carbonate medium and does not grow at pH 7; the maximum pH for growth is more than pH 10, and the optimum pH is 9.5 to 9.7. The optimum NaCl concentration for growth is only 3% (wt/vol) (206–208).

Several alkaliphilic spirochetes were also isolated from Lake Magadi and from Lake Khatyn, Central Asia, by Zhilina et al. Analysis of the genes encoding 16S rRNA indicated a possible fanning out of the phylogenetic tree of spirochetes (207).

Khmelenina et al. (91) isolated two strains of the halotolerant alkaliphilic obligate methanotrophic bacterium *Methylobacter alcaliphilus* sp. nov. from moderately saline soda lakes in Tuva (Central Asia). The strains grow fastest at pH 9.0 to 9.5 and much more slowly at pH 7.0. No growth occurred at pH less than or equal to 6.8. They require $NaHCO_3$ or NaCl for growth in alkaline medium.

TABLE 2. Intracellular pH values in alkaliphilic *Bacillus* strains at different external pH values

Microorganism	External pH	Internal pH
B. alcalophilus	8.0	8.0
	9.0	7.6
	10.0	8.6
	11.0	9.2
B. firmus	7.0	7.7
	9.0	8.0
	10.8	8.3
	11.2	8.9
	11.4	9.6
Bacillus strain YN-2000	7.5	8.5
	8.5	7.9
	9.5	8.1
	10.2	8.4
B. halodurans C-125	7.0	7.3
Intact cells	7.5	7.4
	8.0	7.6
	8.5	7.8
	9.0	7.9
	9.5	8.1
	10.0	8.2
	10.5	8.4
Protoplasts	7.0	7.5
	8.0	7.9
	8.5	8.2
	9.0	8.4
	9.3	8.6

SPECIAL PHYSIOLOGICAL FEATURES OF ALKALIPHILES

Internal pH

Most alkaliphiles have an optimal growth pH at around 10, which is the most significant difference from well-investigated neutrophilic microorganisms. Therefore, the question arises how these alkaliphilic microorganisms can grow in such an extreme environment. Is there any difference in physiological and structural aspects between alkaliphilic and neutrophilic microorganisms? Internal cytoplasmic pH can be estimated from the optimal pH of intracellular enzymes. For example, α-galactosidase from an alkaliphile, *Micrococcus* sp. strain 31-2, had its optimal catalytic pH at 7.5, suggesting that the internal pH is around neutral. Furthermore, the cell-free protein synthesis systems from alkaliphiles optimally incorporate amino acids into protein at pH 8.2 to 8.5, only 0.5 pH unit higher than that of the neutrophilic *Bacillus subtilis* (69).

Another method to estimate internal pH is to measure in cells the inside and outside distribution of weak bases, which are not actively transported by cells. The internal pH was maintained at around 8, despite a high external pH of 8 to 11, as shown in Table 2 (5, 51, 65, 171). Therefore, one of the key features in alkaliphily is associated with the cell surface, which discriminates and maintains the intracellular neutral

environment separate from the extracellular alkaline environment.

Cell Walls

Acidic polymers. Since the protoplasts of alkaliphilic *Bacillus* strains lose their stability in alkaline environments, it has been suggested that the cell wall may play a key role in protecting the cell from alkaline environments. Components of the cell walls of several alkaliphilic *Bacillus* spp. have been investigated in comparison with those of the neutrophilic *B. subtilis*. In addition to peptidoglycan, alkaliphilic *Bacillus* spp. contain certain acidic polymers, such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid (4). The negative charges on the acidic nonpeptidoglycan components may give the cell surface its ability to adsorb sodium and hydronium ions and repulse hydroxide ions and, as a consequence, may assist cells to grow in alkaline environments.

Peptidoglycan. The peptidoglycans of alkaliphilic *Bacillus* spp. appear to be similar to that of *B. subtilis*. However, their composition was characterized by an excess of hexosamines and amino acids in the cell walls compared to that of the neutrophilic *B. subtilis*. Glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and acetic acid were found in hydrolysates. Although some variation in the amide content among the peptidoglycans from alkaliphilic *Bacillus* strains was found, the variation in pattern was similar to that known in neutrophilic *Bacillus* species.

Na^+ Ions and Membrane Transport

Alkaliphilic microorganisms grow vigorously at pH 9 to 11 and require Na^+ for growth (116). The presence of sodium ions in the surrounding environment has proved to be essential for effective solute transport through the membranes of alkaliphilic *Bacillus* spp. According to the chemiosmotic theory, the proton motive force in the cells is generated by the electron transport chain or by excreted H^+ derived from ATP metabolism by ATPase. H^+ is then reincorporated into the cells with cotransport of various substrates. In Na^+-dependent transport systems, the H^+ is exchanged with Na^+ by Na^+/H^+ antiporter systems, thus generating a sodium motive force, which drives substrates accompanied by Na^+ into the cells. The incorporation of a test substrate, α-aminoisobutyrate, increased twofold as the external pH shifted from 7 to 9, and the presence of sodium ions significantly enhanced the incorporation; 0.2 N NaCl produced an optimum incorporation rate that was 20 times the rate observed in the absence of NaCl. Other cations, including K^+, Li^+, NH_4^+, Cs^+, and Rb^+, showed no effect, nor did their counteranions (108).

Mechanisms of Cytoplasmic pH Regulation

The cells have two barriers to reduce pH values from 10.5 to 8 (Fig. 3).

Cell walls containing acidic polymers function as a negatively charged matrix and may reduce the pH value at the cell surface. The surface of the plasma membrane must presumably be kept below pH 9, because the plasma membrane is very unstable at alkaline pH values (pH 8.5 to 9.0) much below the pH optimum for growth.

Plasma membranes may also maintain pH homeostasis by using the Na^+/H^+ antiporter system ($\Delta\psi$ dependent and ΔpH dependent), the K^+/H^+ antiporter, and ATPase-driven H^+ expulsion. Recent studies on the critical antiporters in several laboratories have begun to clarify the number and characteristics of the porters that support active mechanisms of pH homeostasis (53, 76, 109, 114, 130).

My group isolated a nonalkaliphilic mutant strain (mutant 38154) from *B. halodurans* C-125 as the host for cloning genes related to alkaliphily. A 3.7-kb parental DNA fragment (pALK fragment) from the parental strain restored the growth of mutant 38154 at alkaline pH. The transformant was able to maintain an intracellular pH that was lower than the external pH and contained an electrogenic Na^+/H^+ antiporter driven only by $\Delta\psi$ (membrane potential, interior negative). Membrane vesicles prepared from mutant 38154 did not show membrane potential $\Delta\psi$-driven Na^+/H^+ antiporter activity. These results indicate that mutant 38154 affects, either directly or indirectly, electrogenic Na^+/H^+ antiporter activity. This was the first report of a DNA fragment responsible for a Na^+/H^+ antiporter system in the mechanism of alkaliphily (53, 56, 107, 115, 162).

Krulwich et al. (49, 51, 76, 114, 171, 172) have focused their studies on the facultative alkaliphile *Bacillus firmus* OF4, which is routinely grown on malate-containing medium at either pH 7.5 or 10.5. Current work is directed toward clarification of the characteristics and energetics of membrane-associated proteins that must catalyze inward proton movements. One such protein is the Na^+/H^+ antiporter, which enables cells to adapt to a sudden upward shift in pH and to maintain a cytoplasmic pH that is 2 to 2.3 units below the external pH in the most alkaline range of pH for growth. Another is the proton-translocating ATP synthase that catalyzes production of ATP under conditions in which the external proton concentration and the bulk chemiosmotic driving force are low. Three gene loci that are candidates for Na^+/H^+ antiporter, carrying genes with roles in Na^+-dependent pH homeostasis, have been identified. All of them have homologs in *B. subtilis*, in which pH homeostasis can be carried out with either K^+ or Na^+. The physiological importance of one of the *B. firmus* OF4 loci, *nha*C, has been studied by targeted gene disruption, and the same approach is being extended to the others. The *atp* genes, which encode the F_1F_0-ATP synthase of the alkaliphile have interesting motifs in areas of putative importance for proton translocation. As an initial step in studies that will probe the importance and possible role of these motifs, the entire *atp* operon from *B. firmus* OF4 has been cloned and functionally expressed in an *Escherichia coli* mutant with its *atp* genes completely deleted. The transformant does not exhibit growth on succinate but shows reproducible, modest increases in the aerobic growth yields on glucose as well as membrane ATPase activity that exhibits characteristics of the alkaliphile enzyme (114). As a result of these mechanisms, the membrane proteins play a key role in keeping the intracellular pH values in the range between 7.0 and 8.5.

PHYSICAL MAPS OF CHROMOSOMAL DNAs OF ALKALIPHILIC *BACILLUS* STRAINS

How alkaliphiles adapt to their alkaline environments is one of the most interesting and challenging topics that might be clarified by genome analysis. Two physical maps for alkaliphilic *Bacillus* strains have been published: those for *B. firmus* OF4 and *B. halodurans* C-125. A physical map of *B. firmus* OF4 is consistent with a circular chromosome of approximately 4 Mb, with an extrachromosomal element of 110 kb (49, 172). Although analysis is still in progress, several open reading frames for Na^+/H^+ antiporters that may play roles in pH homeostasis have been detected. A physical map of the chromosome of *B. halodurans* C-125 has been constructed to facilitate whole-genome analysis (181, 182). In this strain, the genome size is

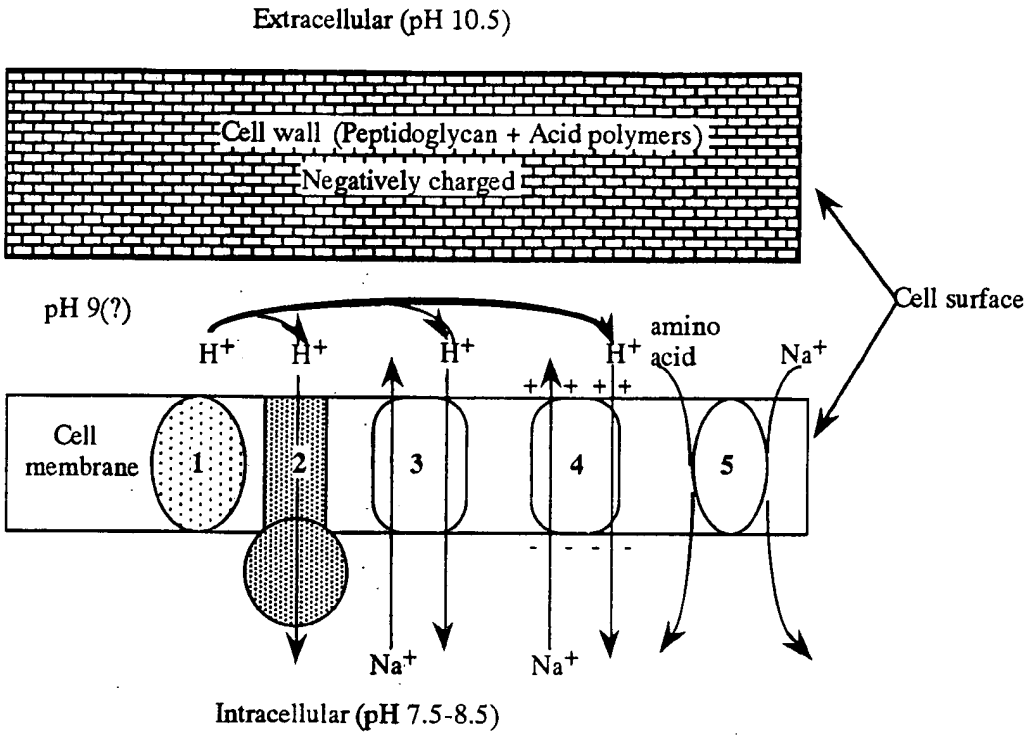

FIG. 3. Schematic representation of cytoplasmic pH regulation.

4.25 Mb. Many open reading frames showing significant similarities to those of *B. subtilis* have been positioned on the physical map. As shown in Fig. 4, the *oriC* regions of the C-125 chromosome have been identificd by Southern blot analysis with a DNA probe containing the *gyrB* region (181, 182).

Whole-genome analysis will inevitably give us useful information about adaptation to alkaline environments.

ALKALINE ENZYMES

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. The first report concerning an alkaline enzyme, published in 1971, described an alkaline protease produced by *Bacillus* sp. strain 221. More than 35 new enzymes have been isolated and purified in my laboratory. Some of these have been produced on an industrial scale.

Alkaline Proteases

In 1971, Horikoshi (66) reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus* sp. strain 221. This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5, and 75% of the activity was maintained at pH 13.0. The enzyme was completely inhibited by diisopropylfluorophosphate or 6 M urea but not by EDTA or *p*-chloromercuribenzoate. The molecular weight of the enzyme was 30,000, which is slightly higher than those of other alkaline proteases. The addition of a 5 mM solution of calcium ions was reflected in a 70% increase in activity at the optimum temperature (60°C). Subsequently, two *Bacillus* strains, AB42 and PB12, were reported which also produced an alkaline protease (9). These strains exhibited a broad pH range (pH 9.0 to 12.0), with a temperature optimum of 60°C for AB42 and 50°C for PB12. Since these reports, many alkaline proteases have been isolated from alkaliphilic microorganisms (35, 146, 187–190). Fujiwara et al. (34) purified a thermostable alkaline protease from a thermophilic alkaliphile, *Bacillus* sp. strain B18. The optimum pH and temperature for the hydrolysis of casein were pH 12 to 13 and 85°C, both of which are higher than those for alkaline proteases. Han and Damodaran (55) reported the purification and characterization of an extracellular endopeptidase from a strain of *Bacillus pumilus* displaying high stability in 10% (wt/vol) sodium dodecyl sulfate and 8 M urea. Some of the enzymes are now commercially available as detergent additives.

Takami et al. (179) isolated a new alkaline protease from alkaliphilic *Bacillus* sp. strain AH-101. The enzyme was most active toward casein at pH 12 to 13 and stable for 10 min at 60°C and pH 5 to 13. The optimum temperature was about 80°C in the presence of 5 mM calcium ions. The alkaline protease showed a higher hydrolyzing activity against insoluble

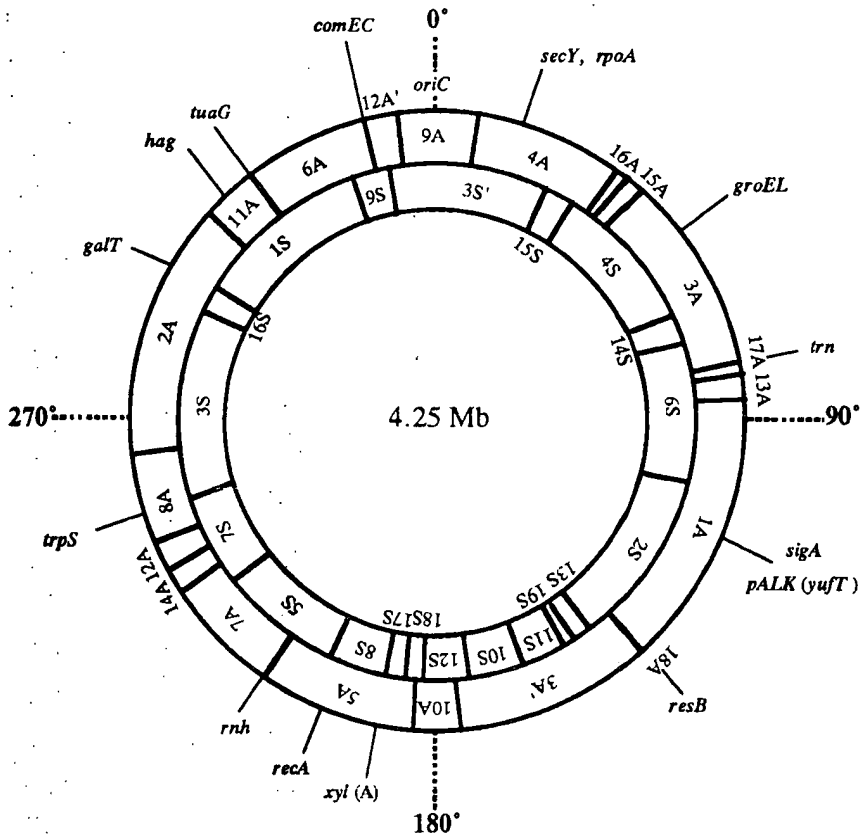

FIG. 4. Physical and genetic map of the chromosome of *B. halodurans* C-125. The outer and inner circles shows the *Asc*I and *Sse*83871 physical maps, respectively. The locations of several genes are indicated on the maps. Dashed lines indicate the approximate positions of the genes.

fibrous natural proteins such as elastin and keratin than did subtilisins and proteinase K (177, 178). Cheng et al. (21) reported a keratinase of a feather-degrading *Bacillus licheniformis* strain, PWD-1. This enzyme was stable from pH 5 to 12. The optimal reaction pHs for feather powder and casein were 8.5 and 10.5 to 11.5, respectively. Zaghloul et al. also reported the isolation, identification, and keratinolytic activity of several feather-degrading bacteria isolated from Egyptian soil. These isolates could degrade chicken feathers (205).

Structural analysis. Schmidt et al. analyzed a series of serine protease genes of alkaliphilic *Bacillus* sp. strain LG12. Four tandem subtilisin-like protease genes were found on a 6,854-bp DNA fragment. The two downstream genes (*sprC* and *sprD*) appear to be transcribed independently, while the two upstream genes (*sprA* and *sprB*) seem to be part of the same transcript (161). Yeh et al. improved the translational efficiency of an alkaline protease YaB gene with different initiation codons in *B. subtilis* and alkaliphilic *Bacillus* YaB (201). Martin et al. found that the solution structure of serine protease PB92 from *B. alcalophilus* presents a well-defined global fold with a flexible structure-binding site. The well-defined global fold was rigid with the exception of a restricted number of sites (123).

Kobayashi et al. isolated and purified an alkaline protease from alkaliphilic *Bacillus* sp. strain KSM-K16 that was suitable for use in detergents (110). Shirai et al. studied the crystal structure of the M protease of alkaliphilic *Bacillus* sp. strain KSM-K16 by X-ray analysis to understand the alkaline adaptation mechanism of the enzyme (164). This analysis revealed a decrease in the number of negatively charged amino acids (aspartic acid and glutamic acid) and lysine residues, and an increase in arginine and neutral hydrophilic amino acids (histidine, asparagine, and glutamine) residues during the course of adaptation.

Other proteases. Several alkaline proteases produced by alkaliphilic actinomycetes have been reported. Tsuchiya et al. (191–193) isolated thermostable alkaline protease from alkaliphilic *Thermoactinomyces* sp. strain HS682. The strain grew in alkaline media between pH 7.5 and 11.5. Maltose gave the highest productivity of protease at a concentration of 10 g/liter. The protease had its maximum proteolytic activity around pH 11.0 and at 70°C. In the presence of Ca^{2+} ions, maximum activity was observed at 80°C. An intracellular alkaline serine protease gene of alkaliphilic *Thermoactinomyces* sp. strain HS682 was cloned and expressed in *E. coli*. Yum et al. (204) purified an extracellular alkaline serine protease produced by *Streptomyces* sp. YSA-130. The optimum temperature and pH for the enzyme activity were 60°C and 11.5, respectively. The enzyme was stable at 50°C and between pH 4 and 12.

A serine protease from the keratin-degrading *Streptomyces pactum* DSM 40530 was purified by casein agarose affinity chromatography by Bockle et al. (15). The proteinase was optimally active in the pH range from 7 to 10 and at temperatures from 40 to 75°C. After incubation with the purified proteinase, rapid disintegration of whole feathers was observed.

Extracellular proteolytic activity was also detected in the haloalkaliphilic archaeon *Natronococcus occultus* as the culture

reached the stationary growth phase (170). Proteolytic activity was precipitated with ethanol and subjected to preliminary characterization. Optimal conditions for activity were attained at 60°C and 1 to 2 M NaCl or KCl. Gelatin zymography in the presence of 4 M betaine revealed a complex pattern of active species with apparent molecular masses ranging from 50 to 120 kDa.

Industrial applications. (i) Detergent additives. The main industrial application of alkaliphilic enzymes is in the detergent industry, and detergent enzymes account for approximately 30% of total worldwide enzyme production. Not all of these are produced by alkaliphilic bacteria. However, many alkaline proteases have been produced by alkaliphilic *Bacillus* strains and are commercially available.

(ii) Dehairing. Alkaline enzymes have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10. These enzymes are commercially available.

(iii) Other applications. An interesting application of alkaline protease was developed by Fujiwara and coworkers (35, 36, 75). They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered. Protease B18′ had a higher optimum pH and temperature, around 13.0 and 85°C. The enzyme was most active toward gelatin on film at pH 10.

Starch-Degrading Enzymes

The first alkaline amylase was produced in Horikoshi-II medium by cultivating alkaliphilic *Bacillus* sp. strain A-40-2 (67). Several types of alkaline starch-degrading enzymes were observed. No alkaline amylases produced by neutrophilic microorganisms have so far been reported. Recent studies revealed that the starch-degrading enzymes α-amylase and cyclomaltodextrin glycosyltransferase (CGTase) are functionally and structurally closely related. α-Amylases have three molecular domains (called A, B, and C), and CGTases contain two additional domains (called D and E).

α-Amylases of alkaliphilic *Bacillus* strains. The alkaline amylase was first produced in alkaliphilic *Bacillus* sp. strain A-40-2 (ATCC 21592), which was selected from about 300 colonies of bacteria grown in Horikoshi-II medium (67). The enzyme is most active at pH 10.0 to 10.5 and retains 50% of its activity between pH 9.0 and 11.5. The enzyme is not inhibited by 10 mM EDTA at 30°C but is completely inactivated by 8 M urea. The enzyme can hydrolyze 70% of starch to yield glucose, maltose, and maltotriose, and it is a type of saccharifying α-amylase. Boyer and Ingle reported an alkaline amylase in strain NRRL B-3881, which was the second report of an alkaline amylase (17, 18). The B-3881 amylase has its optimum pH for enzyme action at 9.2. It yields maltose, maltotriose, and a small amount of glucose and maltotetraose, all of which have a β-configuration. Considerable diversity of α-amylases has been reported. Kim et al. reported that the alkaliphilic *Bacillus* sp. strain GM8901 produced five alkaline amylases in a culture broth (97). McTigue et al. studied the alkaline amylases of three alkaliphilic *Bacillus* strains (126, 127). *Bacillus halodurans* A-59 (ATCC 21591), *Bacillus* sp. strain NCIB 11203, and *Bacillus* sp. strain IMD370 produced alkaline α-amylases with maxima for activity at pH 10.0. Kelly et al. (86) found that the alkaline amylase of *Bacillus* sp. strain IMD370 could hydrolyze raw starch. The enzyme digested raw cornstarch to glucose, maltose, maltotriose, and maltotetraose. The maximum pH for raw starch hydrolysis was pH 8.0, compared to pH 10.0 for soluble starch hydrolysis. It is of interest that degradation of raw starch was stimulated sixfold in the presence of β-cyclodextrin. Lin et al. have recently reported the production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. strain TS-23 (119). Activity staining revealed that two amylases with molecular masses of 150 and 42 kDa were produced. The 42-kDa amylase hydrolyzed only raw starch. Igarashi et al. isolated a novel liquefying α-amylase (LAMY) from cultures of an alkaliphilic *Bacillus* isolate, strain KSM-1378 (73). The enzyme had a pH optimum of 8.0 to 8.5 and displayed maximum activity at 55°C. The structural gene for the amylase contained a single open reading frame 1,548 bp in length, encoding to 516 amino acids that included a signal peptide of 31 amino acids. The four conserved regions were found in the deduced amino acid sequence. Essentially, the sequence of LAMY was consistent with the tertiary structures of reported amylolytic enzymes, which are composed of domains A, B, and C. Furthermore, Igarashi et al. improved the thermostability of the amylase by deleting an arginine-glycine residue in that molecule (74).

Other α-amylases. Kimura and Horikoshi isolated a number of starch-degrading psychrotrophic microorganisms from the environment (102, 104, 106). Recently, a gene coding for a new amylolytic enzyme from *Pseudomonas* sp. strain KFCC 10818 was cloned, and its nucleotide sequence was determined (131). A deduced amino acid sequence contained four highly conserved regions of α-amylases. The haloalkaliphilic *Natronococcus* sp. strain Ah-36 produced an extracellular maltotriose-forming amylase (112). The amylase exhibited maximal activity at pH 8.7 and 55°C in the presence of 2.5 M NaCl. Kobayashi et al. have cloned this α-amylase and expressed it in *Haloferax volcanii* (111).

Cyclomaltodextrin glucanotransferases. Nakamura and Horikoshi discovered many alkaliphilic *Bacillus* strains producing CGTases. The crude enzyme of *Bacillus* sp. strain 38-2 was a mixture of three enzymes: acidic CGTase, having an optimum pH for enzyme action at 4.6, neutral CGTase, having an optimal pH at 7.0, and alkaline CGTase, having an optimal pH at 8.5 (135–138). In 1975, Matsuzawa et al. established the industrial production of cyclodextrin (CD) by using crude CGTase of *Bacillus* sp. strain 38-2 (124). Since then, many alkaliphilic microorganisms producing CGTases have been reported (145, 147). Georganta et al. isolated CGTase-producing psychrophilic alkaliphilic bacteria from samples of deep-sea bottom mud (47). Isolate 3-22 grew at 4°C and showed activity at broad temperature ranges and from pH 5 to 9. The CGTase produced predominantly β-CD, with minor amounts of α- and γ-CDs. The formation of various CDs was observed after accumulation of maltooligosaccharides with degrees of polymerization more than seven (1). Salva et al. isolated 68 CGTase-producing alkaliphilic bacteria from among 400 soil bacteria (157). The enzyme of isolate 76 was partially purified by starch adsorption, and some of its properties were investigated. These properties seem to be quite similar to those of the *Bacillus* sp. strain 38-2 enzyme (135). Chung et al. isolated a thermostable CGTase from *Bacillus stearothermophilus* ET1 (22). The optimum pH for the enzyme-catalyzed reaction was pH 6.0, and the optimum temperature was observed at 80°C. A 13% (wt/vol) cornstarch solution was liquefied and converted to CDs solely by using this enzyme. The cornstarch conversion rate was 44%, and α-, β-, and γ-CDs were produced in the ratio of 4.2:5.9:1. Terada et al. studied the initial reaction of CGTase from the alkaliphilic *Bacillus* sp. strain A2-5a on amylose (186). Cyclic α-1,4-glucans with degrees of polymerization ranging from 9 to more than 60, in addition to α-, β-, and γ-CD, were detected at early stages of enzymatic reactions. Subsequently, large cyclic α-1,4-glucans were converted into smaller cyclic α-1,4-glucans. The final major product was β-CD. From the

industrial point of view, γ-CD is of practical interest because it is rare and can encapsulate larger compound molecules. Recently, Parsiegla et al. reported that mutation of *Bacillus circulans* 8 was effective in increasing γ-CD production (154). Yamane's group has extensively investigated the molecular structure of the CGTase of alkaliphilic *Bacillus* sp. strain 1011 (91–101, 113, 133, 134). Kimura et al. isolated the enzyme, purified and cloned its gene (99, 100). The enzyme, consisting of 686 amino acid residues, was crystallized and subjected to X-ray analysis. The molecule consists of five domains, designated A to E, and its backbone structure is similar to the structure of other bacterial CGTases. The molecule has two calcium-binding sites where calcium ions are coordinated by seven ligands, forming a distorted pentagonal bipyramid. Three histidine residues in the active center of CGTase participate in the stabilization of the transition state. His-327 is especially important for catalysis over the alkaline pH range (133). Analysis of the three-dimensional structures of CGTases has revealed that four aromatic residues, which are highly conserved among CGTases but not in α-amylases, are located in the active center (134).

Industrial production of cyclodextrins. In 1969, Corn Products International Co. in the United States began producing β-CD by using *B. macerans* CGTase. Teijin Ltd. in Japan also produced β-CD by using the *B. macerans* enzyme in a pilot plant. However, there were two serious problems in both production processes: (i) the yield of CD from starch was not high, and (ii) Toxic organic solvents such as trichloroethylene, bromobenzene, and toluene were used to precipitate CD owing to the low conversion rate. The use of CGTase of alkaliphilic *Bacillus* sp. strain 38-2 overcame all these weak points and led to the mass production of crystalline α-, β-, γ-CD at low cost without using any organic solvents. The yield of CD was 85 to 90% from amylose and 70 to 80% from potato starch on a laboratory scale. Owing to the high conversion rate, CDs could be directly crystallized from the hydrolysate of starch without the addition of organic solvents (124). These simple methods reduced the cost of β-CD from $1,000/kg to $5/kg and that of a α-CD to within $15/kg. This has paved the way for its use in large quantities in foodstuffs, chemicals, and pharmaceuticals. Several other processes to produce CDs have been developed since the original report by Matsuzawa et al. (124). Kim et al. reported an enzymatic production of CDs from milled corn starch in an ultrafiltration membrane bioreactor to reduce product inhibition (96). In a batch operation with ultrafiltration, the conversion yield was increased 57% compared with that without ultrafiltration. Okada et al. also developed a bioreactor system with the enzyme immobilized on a capillary membrane (148). The percentage of CDs to total sugar obtained was slightly more than 60% under most operating conditions. Abraham immobilized CGTase for the continuous production of CDs (2). The immobilized enzyme had a pH optimum shifted to the alkaline side (from 6.5 to 7.5) and had a reduced temperature optimum (from 60°C to 50–55°C). Lima et al. developed a unique CD production process (118). A 10% (wt/vol) solution of cassava starch, liquefied with α-amylase, was incubated with CGTase by using only the enzyme and added ethanol (from 1 to 5%), followed by the addition of the yeast *Saccharomyces cerevisiae* (12% [wt/vol]) plus nutrients. However, the production costs were not reported.

Pullulanases. In 1976, Nakamura et al. discovered an alkaline pullulanase of *Bacillus* sp. strain 202-1 (139). The enzyme has a pH optimum for enzyme action at pH 8.5 to 9.0 and is stable for 24 h at pH 6.5 to 11.0 at 4°C. The enzyme is most active at 55°C and is stable up to 50°C for 15 min in the absence of substrate. Kelly et al. found that alkaliphilic *B. halodurans* A-59 (ATCC 21591) produced three enzymes, α-amylase, pullulanase, and α-glucosidase, in culture broth (87). These three enzymes were separately produced, and the levels of α-glucosidase and pullulanase reached their maxima after 24 h of cultivation at the initial pH 9.7. Although this pullulanase was not purified, the indicated pH optimum was at 7.0.

Two highly alkaliphilic pullulanase-producing bacteria were isolated from Korean soil (94, 95). The two isolates were extremely alkaliphilic, since bacterial growth and enzyme production occurred at pH values ranging from pH 6.0 to 12.0 for *Micrococcus* sp. strain Y-1 and pH 6.0 to 10.0 for *Bacillus* sp. strain S-1. The enzyme displayed a temperature optimum of around 60°C and a pH optimum of around pH 9.0. The extracellular enzymes of both bacteria were alkaliphilic and moderately thermoactive; optimal activity was detected at pH 8.0 to 10.0 and between 50 and 60°C.

In screening alkaline cellulases from detergent additives, Ara and coworkers isolated a novel alkaline pullulanase from alkaliphilic *Bacillus* sp. strain KSM-1876, which was identified as a relative of *Bacillus circulans* (8, 72). The enzyme had an optimum pH for enzyme action of around 10.0 to 10.5. This enzyme is a good candidate for use as an additive to dishwashing detergents. Ito's group then found another alkaline amylopullulanase from alkaliphilic *Bacillus* sp. strain KSM-1378 (7). This enzyme efficiently hydrolyzed the α-1,4 and α-1,6 linkages of amylose, amylopectin, and glycogen at alkaline pH values. The kinetic studies revealed two independent active sites for the α-1,4 and α-1,6 hydrolytic reactions. Incubation of the enzyme at 40°C and pH 9.0 caused complete inactivation of the amylase activity within 4 days, but the pullulanase activity remained at the original level under the same conditions (7). This alkaline amylopullulanase can therefore be considered to be a "two-headed" enzyme molecule. Limited proteolysis with papain also revealed that the α-1,6- and α1,4 hydrolytic activities were associated with two different active sites (6). Furthermore, analysis of the amino acid sequence and molecular structure of the enzyme showed two different active sites (57). The enzyme was observed by transmission electron microscopy; it appeared to be a bent dumbbell-like molecule with a diameter of approximately 25 nm.

Takagi et al. reported diversity in the size and alkaliphily of thermostable α-amylase-pullulanases produced by recombinant *Escherichia coli*, *B. subtilis* and the wild-type *Bacillus* sp. strain XAL601 (176). It was revealed that the noncatalytic C-terminal region may be responsible for the high optimum pH of the enzyme activity. These observations were also reported for CGTase (82, 83) and alkaline carboxymethyl cellulase (CMCase) (132).

Lin et al. purified a thermostable pullulanase from the thermophilic alkaliphilic *Bacillus* sp. strain TS-23 (120). This purified enzyme had both pullulanase and amylase activities. The temperature and pH optima for both pullulanase and amylase activities were 70°C and pH 8 to 9, respectively. The enzyme remained more than 96% active at temperatures below 65°C, and both activities were retained at temperatures up to 90°C in the presence of 5% sodium dodecyl sulfate (120).

In some food industries, enzymes showing activity at lower temperatures have been requested for use in food processing. Psychrotrophic bacteria are thought to be potential producers of these enzymes. Kimura and Horikoshi (103, 105) reported that an alkalipsychrotrophic strain, *Micrococcus* sp. strain 207, extracellularly produced amylase and pullulanase. The pullulanase was purified to an electrophoretically homogeneous state by conventional methods. The purified enzyme was free of α-amylase activity. It had a pH optimum at 7.5 to 8.0 and was relatively thermostable (up to 45°C). The enzyme could

hydrolyze the α-1,6-linkages of amylopectins, glycogens, and pullulan. Although many alkaline pullulanases have been reported as described above, no industrial application has been developed yet.

Cellulases

Alkaline cellulases of alkaliphilic *Bacillus* strains. Commercially available cellulases display optimum activity over a pH range from 4 to 6. No enzyme with an alkaline optimum pH for activity (pH 10 or higher) had been reported before the rediscovery of alkaliphiles. Horikoshi and coworkers found bacterial isolates (*Bacillus* sp. strains N4 and 1139) producing extracellular alkaline CMCases (37, 71). One of these, alkaliphilic *Bacillus* sp. strain N-4 (ATCC 21833), produced multiple CMCases that were active over a broad pH range (pH 5 to 10). Sashihara et al. cloned the cellulase genes of *Bacillus* sp. strain N-4 in *E. coli* HB101. Several cellulase-producing clones with different DNA sequences were obtained (159). Another bacterium, *Bacillus* sp. strain 1139, produced one CMCase, which was purified and shown to have optimum pH for activity at pH 9.0. The enzyme was stable over the pH range from 6 to 11 (for 24 h at 4°C and 10 min at up to 40°C). The CMCase gene of *Bacillus* sp. strain 1139 was also cloned in *E. coli* (38–42). Beppu's group (132, 153) constructed many chimeric cellulases from *B. subtilis* and *Bacillus* sp. strain N-4 enzyme genes in an effort to understand the alkaliphily of N-4 enzymes. Although the genes have high homology, the pH activity profiles of the two enzymes are quite different; the *B. subtilis* enzyme (BSC) has its optimum pH at 6 to 6.5, whereas the *Bacillus* sp. strain N-4 enzyme (NK1) is active over a broad pH range from 6 to 10.5. The chimeric cellulases showed various chromatographic behaviors, reflecting the origins of their C-terminal regions. The pH activity profiles of the chimeric enzymes in the alkaline range could be classified into either the BSC or NK1 type, depending mainly on the origins of the fifth C-terminal regions.

Cellulases as laundry detergent additives. The discovery of alkaline cellulases created a new industrial application of cellulase as a laundry detergent additive. Ito (79a) mixed alkaline cellulases with laundry detergents and studied their effect by washing cotton underwear. The best results were obtained by one of the alkaline cellulases produced by an alkaliphilic *Bacillus* strain. However, the yield of enzyme was not sufficient for industrial purposes. Ito and coworkers (79, 203) then isolated the alkaliphilic *Bacillus* sp. strain KSM-635 from the soil and succeeded in producing an alkaline cellulase as a laundry detergent additive on an industrial scale.

Besides the KSM-635 enzyme, Shikata et al. isolated three strains, alkaliphilic *Bacillus* strains KSM-19, KSM-64, and KSM-520, producing alkaline cellulases for laundry detergents (163). The activities of these enzymes (pH optima; 8.5 to 9.5) were not inhibited at all by metal ions or various components of laundry products, such as surfactants, chelating agents, and proteinases. With a view to increasing industrial production, Sumitomo et al. (173, 174) overexpressed the alkaline cellulase of alkaliphilic *Bacillus* sp. strain KSM-64 by using *B. subtilis* harboring the vector pHSP64 carrying the cellulase gene. By this process, they produced 30 g of alkaline cellulase in 1 liter. After the discovery of the industrial application of alkaline cellulase as a detergent additive, many microbiologists have extensively studied alkaline cellulases (52, 58, 77, 93, 129, 158). Further details are reviewed by Ito (78).

Alkaline cellulases from other alkaliphiles. Park et al. (152) and Damude et al. (24) studied a semialkaline cellulase produced by alkaliphilic *Streptomyces* strain KSM-9. Dasilva et al. (25) reported two alkaliphilic microorganisms, *Bacillus* sp. strain B38-2 and *Streptomyces* sp. strain S36-2. The optimum pH and temperature of the crude enzyme activities ranged from 6.0 to 7.0 at 55°C for the *Streptomyces* strain and 7.0 to 8.0 at 60°C for the *Bacillus* strain. However, the results indicated that the properties of these enzymes were not appropriate for industrial purposes.

Lipases

Although the initial motivation for studying alkaline lipase was its application to detergents, many alkaline lipases were significantly inhibited in the presence of either alkylbenzene sulfate or dodecyl benzene sulfonate.

Watanabe et al. (197) conducted an extensive screening for alkaline lipase-producing microorganisms from soil and water samples. Two bacterial strains were selected as potent producers of alkaline lipase. These were identified as *Pseudomonas nitroreducens* nov. subsp. *thermotolerans* and *P. fragi*. The optimum pH of the two lipases was 9.5. Both enzymes were inhibited by bile salts such as sodium cholate, sodium deoxycholate, and sodium taurocholate at 0.25%. However, further work has not been reported.

A thermophilic lipase-producing bacterium was isolated from a hot-spring area of Yellowstone National Park (196). The organism, characterized as a *Bacillus* sp., grew optimally at 60 to 65°C and in the pH range from 6 to 9. The partially purified lipase preparation had an optimum temperature of 60°C and an optimum pH of 9.5. It retained 100% of the original activity after being heated at 75°C for 30 min. It was active on triglycerides containing fatty acids having a carbon chain length of $C_{16:0}$ to $C_{22:0}$, as well as on natural fats and oils.

Bushan et al. (12) found a lipase produced from an alkaliphilic *Candida* species in a solid-state fermentation. The lipase from this microorganism had temperature and pH optima of 40°C and 8.5, respectively, and was stable at 45°C for 4 h. The enzyme activity was stimulated by Ni^{2+} and Ca^{2+} ions but inhibited by Fe^{2+} and Fe^{3+} ions.

Xylanases

The first paper describing the isolation of a xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (70). The purified enzyme of *Bacillus* sp. strain C-59-2 exhibited a broad optimal pH ranging from 6.0 to 8.0. In the culture broth of *Bacillus halodurans* C-125, two xylanases were found (64). Xylanase A had a molecular weight of 43,000, and xylanase N had a molecular weight of 16,000. Xylanase N was most active at pH 6 to 7, and xylanase A was most active pH 6 to 10 and had some activity at pH 12. The xylanase A gene was cloned, sequenced, and expressed in *E. coli* (59–63). Four thermophilic alkaliphilic *Bacillus* strains (W1 [JCM2888], W2 [JCM2889], W3, and W4) produced xylanases (149, 150). The pH optima for enzyme action of strains W1 and W3 was 6.0, and that for strains W2 and W4 was between 6 and 7. The enzymes were stable between pH 4.5 and 10.5 at 45°C for 1 h. The optimum temperatures of xylanases of W1 and W3 were 65°C, and those of W2 and W4 were 70°C. The degree of hydrolysis of xylan was about 70% after 24 h of incubation.

Since the demonstration that alkali-treated wood pulp could be biologically bleached by xylanases instead of by the usual environmentally damaging chemical process involving chlorine, the search for thermostable alkaline xylanases has been extensive. Dey et al. isolated an alkaliphilic thermophilic *Bacillus* strain (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50°C (27). Khasin et al. reported

that alkaliphilic *B. stearothermophilus* T-6 produced an extracellular xylanase that optimally bleached pulp at pH 9 and 65°C (90). Nakamura et al. also reported that an alkaliphilic *Bacillus* strain, 41M-1, isolated from soil, produced multiple xylanases extracellularly (140, 142, 143). One of the enzymes, xylanase J, was most active at pH 9.0. The optimum temperature for the activity at pH 9.0 was around 50°C. Then, an alkaliphilic and thermophilic *Bacillus* strain, TAR-1, was isolated from soil (141). The xylanase was most active over a pH range of 5.0 to 9.5 at 50°C. Optimum temperatures of the crude xylanase preparation were 75°C at pH 7.0 and 70°C at pH 9.0. These xylanases did not act on cellulose, indicating a possible application of the enzyme in biological debleaching processes.

Blanco et al. reported that an enzyme from *Bacillus* sp. strain BP-23 facilitated the chemical bleaching of pulp, generating savings of 38% in terms of chlorine dioxide consumption (14). Recently, Garg et al. reported a biobleaching effect of *Streptomyces thermoviolaceus* xylanase on birchwood kraft pulp. *S. thermoviolaceus* xylanase had the advantage of activity and stability at 65°C (43, 44).

Subsequently, many thermostable alkaline xylanases have been produced from various alkaliphiles isolated from geothermal areas (28, 122, 175).

Pectinases

The first paper on alkaline endopolygalacturonase produced by alkaliphilic *Bacillus* sp. strain P-4-N was published in 1972 (68). The optimum pH for enzyme action was 10.0 for pectic acid. Fogarty and coworkers (33, 85) then reported that *Bacillus* sp. strain RK9 produced endopolygalacturonate lyase. The optimum pH for the enzyme activity toward acid-soluble pectic acid was 10.0. Subsequently, several papers on potential applications of alkaline pectinase have been published. The first application of alkaline pectinase-producing bacteria in the retting of Mitsumata bast was reported by Yoshihara and Kobayashi (202). The pectic lyase (pH optimum 9.5) produced by the alkaliphilic *Bacillus* sp. strain GIR 277 has been used in improving the production of a type of Japanese paper. A new retting process produced a high-quality, nonwoody paper that was stronger than the paper produced by the conventional method. Tanabe et al. tried to develop a new waste treatment by using the alkaliphilic *Bacillus* sp. strain GIR 621-7 (184, 185). Cao et al. isolated four alkaliphilic bacteria, NT-2, NT-6, NT-33, and NT-82, producing pectinase and xylanase (20). One strain, NT-33, had an excellent capacity for degumming ramie fibers.

Chitinases

Tsujibo et al. isolated chitinases from the alkaliphilic *Nocardiopsis albus* subsp. *prasina* OPC-131 (195). The isolate produced two types of chitinases. The optimum pH of chitinase A was pH 5.0, and that of chitinase B was pH 7.0. Recently, Bhushan and Hoondal isolated the alkaliphilic, chitinase-producing *Bacillus* sp. strain BG-11 (13). The purified chitinase exhibited broad pH and temperature optima of 7.5 to 9.0 and 45 to 55°C, respectively. The chitinase was stable between pH 6.0 and 9.0 at 50°C for more than 2 h. Ag^+, Hg^{2+}, dithiothreitol, β-mercaptoethanol, glutathione, iodoacetic acid, and iodoacetamide inhibited the activity up to 50%. No further studies have been reported.

METABOLITES PRODUCED BY ALKALIPHILES

2-Phenylamine

Hamasaki et al. found that a large amount of 2-phenylethylamine was synthesized by cells of the alkaliphilic *Bacillus* sp. strain YN-2000. This amine was secreted in the medium during cell growth (54).

Carotenoids of Alkaliphilic *Bacillus* Strains

Aono and Horikoshi reported that alkaliphilic *Bacillus* sp. strains A-40-2, 2B-2, 8-1, and 57-1 produce yellow pigments in the cells (3) and that these are triterpenoid carotenoids. However, A-59, M-29, and Y-25, white strains when grown in Horikoshi-II medium, did not produce carotenoids. The physiological role of the yellow pigments was not reported.

Siderophores

Gascoyne et al. isolated a siderophore-producing alkaliphilic bacterium that accumulated iron, gallium, and aluminum (45, 46). Enrichment cultures initiated with samples from a number of alkaline environmental sources yielded 10 isolates. From this group, selections were made on the basis of growth at high pH and the gallium-binding capacity of the siderophores. It was found that some isolates grew well and high concentrations of siderophore were detected whereas others grew well in the presence of much lower concentrations of siderophore. The effect of iron, gallium, and aluminum on growth and siderophore production in batch culture was investigated for six isolates. The presence of iron greatly decreased the siderophore concentration in these cultures, whereas the response to added gallium or aluminum was dependent upon the isolate.

Cholic Acid Derivatives

Kimura et al. isolated an alkaliphilic *Bacillus* strain from soil that grew well in media containing cholic acid (CA) at 5% (wt/vol) or higher concentrations (98). The 7α- and 12α-hydroxyl groups of CA were efficiently converted to keto groups, with the conversion rate for both hydroxyl groups reaching 100% by 72 h of cultivation. The strain also converted a 3α-hydroxyl group to a keto group, but the conversion rate was about 5% at 72 h. The strain neither affected any other part of the CA molecule nor oxidized 7β- or 12β-hydroxyl groups. By NTG mutagenesis, they isolated five mutants that selectively produced the following compounds from CA at high yield (close to 100%): strains M-4 and M-5 produced 7,12-diketolithocholic acid, strain 250 produced 7-ketodeoxycholic acid, and strains 124 and 336 yielded ketochenodeoxycholic acid. Furthermore, strains M-4, M-5, and 250 produced only 7-ketolithocholic acid from chenodeoxycholic acid.

Organic Acids

During the cultivation of alkaliphiles, the pH values of the culture media often decrease sharply due to the production of organic acids, which are produced by growth on carbohydrates. Paavilainen et al. reported comparative studies of organic acids produced by alkaliphilic bacilli (151). Four bacilli, *Bacillus* sp. strain 38-2 (ATCC 21783), *B. alkalophilus* subsp. *halodurans* (ATCC 27557), *B. alcalophilus* (ATCC 27648), and *Bacillus* sp. strain 17-1 (ATCC 31007), were cultured in the presence of various 1% (wt/vol) sugars and related compounds such as sugar alcohols. All these alkaliphiles produced acetic acid (4.5

to 5 g/liter at the maximum), while formic acid was produced by only one of the strains. In contrast to neutrophilic bacilli, acetoin, butanediol, and ethanol were not detected. Moderate amounts of isobutyric, isovaleric, α-oxoisovaleric, α-oxo-β-methylvaleric, α-oxoisocaproic, and phenylacetic acids were generated by three of the alkaliphiles.

Antibiotics and Enzyme Inhibitors

Since alkaliphiles were rediscovered, many Japanese pharmaceutical companies have tried using alkaline media to isolate new microorganisms producing new antibiotics. Although several have been found and reported, none are as yet commercially available.

The first report was published in 1980 by Sato et al. (160), where they recorded the isolation of *Paecilomyces lilacinus* 1907 from soil by using an alkaline medium (pH 10.5). New antibiotics were produced only under alkaline conditions (pH 9 to 10.5). *P. lilacinus* 1907 produced a major product, 1907-II, and a minor product, 1907-VIII, which had antibacterial and antifungal activities.

Subsequently, an alkaliphilic *Nocardiopsis dassonvillei* strain, OPC-15, that produced phenazine antibiotics under alkaline culture conditions was isolated (194). Strain OPC-15 grew well at pH 10 and accumulated two phenazine antibiotics in the mycelia, 1,6-dihydroxyphenazine (I) and 1,6-dihydroxyphenazine 3,5-dioxide (III), at different growth temperatures. Antibiotic I was formed when the organism was cultured at 27°C for 6 to 8 days, whereas the antibiotic III was formed only when the organism was grown at 27°C for 6 days and incubated further at 4°C for 2 days.

Bahn et al. isolated a novel aldose reductase inhibitor, YUA001, from the alkaliphilic *Corynebacterium* sp. strain YUA25-1. Compound YUA001 was purified from the supernatant of culture broth by successive silica gel column chromatography steps. The molecular formula of YUA001 was determined to be $C_{13}H_{19}NO_2$. The compound had no antimicrobial activity against some gram-positive and gram-negative bacteria, fungi, and yeasts (10).

Besides these antibiotics, many compounds have been found and screened by pharmaceutical companies. However, there is one weak point in the direct production of antibiotics by alkaliphilic microorganisms. Many antibiotics are very unstable under alkaline condition, and it is highly possible that antibiotics produced are being destroyed during cultivation. However, some alkaliphiles, especially actinomycetes, can grow in neutral media under specified conditions, offering a strategy for the recovery of bioactive compounds.

FUTURE OF ALKALIPHILES

Since the rediscovery of alkaliphilic bacteria, more than 1,000 papers on many aspects of alkaliphiles and alkaliphily have been published. The alkaliphiles are unique microorganisms, with great potential for microbiology and biotechnological exploitation. The aspects that have received the most attention in recent years include (i) extracellular enzymes and their genetic analysis, (ii) mechanisms of membrane transport and pH regulation, and (iii) the taxonomy of alkaliphilic microorganisms. What will be the next line of development? It is unclear, but it may be the wider application of enzymes. Alkaline enzymes should find additional uses in various fields of industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents. Furthermore, alkaliphiles may be very good general genetic resources for such applications as production of signal peptides for secretion and promoters for hyperproduction of enzymes.

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Replacement of the *Bacillus subtilis* Subtilisin Structural Gene with an In Vitro-Derived Deletion Mutation

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The entire subtilisin structural gene from *Bacillus subtilis* 1168 has been cloned, and its nucleotide sequence has been determined. When expressed on a high-copy-number shuttle vector, a fivefold increase in serine protease activity was observed. The DNA sequence of the gene is 80% homologous to the *Bacillus amyloliquefaciens* subtilisin structural gene, and the translated mature coding sequence is 85% homologous to the published protein sequence of subtilisin BPN'. The chloramphenicol resistance determinant of a plasmid integrated at the subtilisin locus was mapped by PBS1 transduction and was found to be linked to *glyB* (83%) and *argC* (60%), but not with *metC* or *purB*. The chromosomal locus containing the wild-type subtilisin allele was replaced with an in vitro-derived allele of the gene (*Δapr-684*) that contained a 684-base-pair deletion. The technique used for introducing the deletion is a variation of the gene replacement methods used in *Saccharomyces cerevisiae* and *Escherichia coli*. When used in *B. subtilis*, deletion mutants could be directly screened among the transformants. Physiological characterization of the *Δapr-684* mutation revealed no discernable effect on the formation of heat-resistant endospores, but strains carrying the mutation produced only 10% of wild-type serine protease activity. A model is presented that outlines the pathway for plasmid integration and deletion formation in *B. subtilis*.

Under conditions of nutrient deprivation, *Bacillus subtilis* 1168 initiates a developmental program from which heat-resistant endospores are produced. Closely associated with this temporal and sequential order of gene expression is the appearance of an esterase and at least two proteases in the supernatant of the culture. The two best-characterized proteases are the neutral protease, a metalloenzyme sensitive to EDTA, and an alkaline protease (subtilisin), a serine protease sensitive to phenylmethylsulfonyl fluoride (17, 18, 26, 44).

Considerable controversy has been generated over whether these hydrolytic enzymes have a role in the normal development of the endospore or act simply as scavenging enzymes when usable nutrients have been depleted from the medium (14, 31). Mutations that block sporulation at the earliest stage of development (stage 0 mutations) are pleiotropic and result in the secretion of little or no subtilisin into the culture supernatant. Analysis of mutations in these *spo0* loci has resulted in the suggestion by some investigators that the expression of extracellular subtilisin is required for normal sporulation (14, 31). Many of the subtilisin-deficient mutants that have been isolated are pleiotropic and are blocked at an early stage in sporulation (17, 18). Shoer and Rappaport (39) characterized a *B. subtilis* 1168 serine protease mutant and isolated an extracellular protein that was shown to be a fragment of the wild-type enzyme. Further characterization showed only 1 in 53 molecules to be functional, but the strain carrying the mutant allele sporulated normally (39). The chromosomal map location of this mutation was not determined. Mutations have been isolated in the structural gene of the neutral protease (*nprE* locus), and studies have shown that a functional gene product is not required for normal sporulation (26, 44).

In this paper, the structural gene of *Bacillus amyloliquefaciens* subtilisin was used as a hybridization probe to isolate the *B. subtilis* 1168 subtilisin gene. The nucleotide sequence of the *B. subtilis* subtilisin gene was determined, and the map location was determined by integrating a chloramphenicol-resistant plasmid at the subtilisin locus. This particular method of mapping cloned genes in *B. subtilis* is a widely accepted technique (9, 13). The chromosomal locus containing the subtilisin gene was then replaced in vivo with an in vitro-derived version of the gene that contained a 684-base-pair (bp) deletion. The technique used has been termed gene replacement and is a variation of methods reported for use in *Saccharomyces cerevisiae* and *Escherichia coli* (12, 36, 40). Physiological characterization of the subtilisin deletion mutation in *B. subtilis* 1168 revealed no discernible effect on endospore development. However, strains carrying the deletion mutations produced only 10% of wild-type serine protease activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *E. coli* MM294 was cultured in L broth and transformed by the procedure of Dagert and Ehrlich (6). *B. subtilis* strains were cultured on tryptose-blood agar base (Difco Laboratories) or minimal glucose medium and were transformed by the procedure of Anagnostopoulos and Spizizen (2). The preparation of PBS1 transducing lysates and PBS1 transduction and mapping have been described elsewhere (15). The cloning vector used in this study is pBS42, an *E. coli-B. subtilis* shuttle vector (4). The plasmid consists of the pBR322 origin of replication, the pUB110 origin of replication, and the chloramphenicol acetyl transferase gene from pC194, a *Staphylococcus aureus* plasmid. The plasmid pJH101 (10) was kindly provided by J. Hoch. The plasmid pS4 contained the subtilisin structural gene from *B. amyloliquefaciens* and was kindly provided by J. Wells (46).

Media and reagents. Bacterial alkaline phosphatase, *Kpn*I, and *Hin*cII were from Bethesda Research Laboratories, Inc. *Pvu*I, *Pvu*II, *Eco*RI, *Nco*I, T4 DNA ligase, and the large fragment of DNA polymerase I were from New England

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TABLE 1. Designation, genotype, and origin of strains of *B. subtilis*

Strain	Relevant genotype	Origin
1168	*trpC2*	J. A. Hoch
JH703	*trpC2 pheA12 Δspo0A677*	J. A. Hoch
IA84	*glyB33 metD1*	BGSC[a]
IA151	*argC4 hisA1 phe-1 catA*	BGSC
BG16	*purB6 metB5 leuA8 lys-21 hisA thr-5 sacA321*	A. Galizzi (PB 1665)
BG31	*glyB133 hisH metA29 thi-78 recH342*	Prozorov
BG77	*trpC2 prt-77*	NTG × 1168[b]
BG81	*metB5 prt-77*	BG16 DNA × BG77[c]
BG84	*Δspo0A677 prt-77*	JH703 DNA × BG81
BG2014	*trpC2 apr-1*::pIV6	pIV6 DNA × 1168[d]
BG2015	*glyB133 hisH metA29 thi-78 recH342 apr-1*::pIV6	pIV6 DNA × BG31
BG2016	*trpC2 prt-77 apr*::pIDV1.4	pIDV1.4 DNA × BG77
BG2017	*trpC2 prt-77 Δapr-684*::pIDV1.4	pIDV1.4 DNA × BG77
BG2018	*trpC2 prt-77 Δapr-684*	Cm[s] of BG2017[e]
BG2019	*Δapr-684*	BG2018/PBS1 × IA84
BG2020	*argC4 hisA1 phe-1 catA apr-1*::pIV6	pIV6 DNA × IA151[f]

[a] Bacillus Genetic Stock Center, Columbus, Ohio.

[b] The protease deficient mutation, *prt-77*, was obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of strain 1168. The phenotype of this mutant is explained in the text.

[c] Strain BG77 was transformed with BG16 DNA.

[d] When the plasmid pIV6 was transformed into strain BG77, a protease-deficient phenotype was observed (data not shown). In theory this plasmid should interrupt the subtilisin-coding sequence upon integration; consequently all strains transformed with this plasmid were assigned a genotypic designation of *apr-1*::pIV6.

[e] A spontaneous chloramphenicol-sensitive derivative of strain BG2017 was isolated by growth of the culture in the absence of antibiotic selection.

[f] Strain IA84 was transduced with a PBS1 lysate made from strain BG2018.

Biolabs. Selection for plasmid transformants in *E. coli* was on LB agar containing 12.5 μg of chloramphenicol per ml. Plasmid transformants of *B. subtilis* were selected on tryptose-blood agar base or LB plus skim milk agar supplemented with 5 μg of chloramphenicol per ml. LB plus skim milk agar contained 1.5% (wt/wt) Carnation powdered nonfat milk. LB plus starch agar contained 1% (wt/wt) soluble starch (Sigma Chemical Co.). Serine protease activity was assayed in solution by measuring the change in adsorbance at 412 nm per minute upon incubation with 0.2 mg of succinyl(-L-Ala-L-Ala-L-Pro-L-Phe)*p*-nitroanilide (Vega) per ml in 0.1 M sodium phosphate (pH 8) at 25°C (7, 46). Cultures were grown to the late-logarithmic phase of growth in modified Schaeffer medium (17); upon continued incubation, samples were removed every 2 h to assay supernatants for subtilisin activity. The percentage of mature endospores was determined from dual platings on tryptose-blood agar base plates before and after heating samples of the culture for 10 min at 80°C. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis was performed by the method of Adelberg et al. (1).

Physical characterization of the cloned DNA. Plasmid DNA was prepared from *E. coli* transformants by the alkaline lysis method of Birnboim and Doly (5). *B. subtilis* chromosomal DNA was prepared by the method of Marmur (22). DNA fragments from restriction enzyme digests were resolved and analyzed by electrophoresis on 1% agarose or 6% polyacrylamide gels. Restriction fragments to be sequenced were ligated into appropriate sites of M13 phage vectors mp8 or mp9 (25). DNA sequencing was carried out by dideoxy methods (35). DNA probes were labeled with [α-^{32}P]CTP by nick translation (32). For Southern hybridization analysis, digested DNA fragments were separated on 1% agarose and depurinated as described by Wahl et al. (45). The DNA was transferred to nitrocellulose by the method of Southern (41). Hybridization and washings were performed as described by Maniatis et al. (20).

RESULTS

Isolation of protease-deficient mutants. To facilitate selection of plasmids containing protease genes, a strain that was deficient in protease production was constructed. Strain 1168 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and plated on skim milk plates. Colonies producing a smaller halo were picked for further analysis. Each colony was characterized for protease production on skim milk plates and amylase production on starch plates. One such isolate, which was partially protease deficient (Prt$^-$), amylase positive, and sporulation positive, was designated BG77, and the mutation was designated *prt-77*. The *prt-77* allele was placed in a *spo0A* background by congression (Table 1). This strain, BG84, was completely devoid of protease activity on skim milk plates.

Cloning and expression. Southern blot analysis of *B. subtilis* 1168 chromosomal DNA digested with *Eco*RI showed that a single 6-kilobase (kb) *Eco*RI fragment hybridized with a labeled fragment from the *B. amyloliquefaciens* subtilisin gene (46; J. Wells, unpublished results). Slices in the 6-kb range were cut from a preparative agarose gel of *Eco*RI-digested 1168 DNA. The DNA was electroeluted and ligated into pBS42 that had been digested with *Eco*RI and treated with bacterial alkaline phosphatase. Amplification of the ligation mixture was accomplished by transforming *E. coli* MM294 and isolating plasmid DNA from a suspension of 5,000 pooled colonies. This plasmid pool was transformed into *B. subtilis* BG84 (Table 1), a protease-deficient strain, and protease overproducing colonies were screened by plating on LB agar plus skim milk. Plasmid DNA was isolated from protease overproducing colonies and examined by Southern analysis for a 6-kb *Eco*RI insert that hybridized to a fragment from the C terminus of the subtilisin structural gene from *B. amyloliquefaciens*. A positive clone was identified by hybridization, and the plasmid was designated pS168.1. *B. subtilis* BG84 transformed with pS168.1 secreted serine protease at a level fivefold over that produced in *B. subtilis* 1168. *B. subtilis* BG84 does not produce detectable levels of serine protease when assayed with the specific chromogenic substrate as described above. The addition of EDTA to the supernatants did not affect the assay results, but the addition of phenylmethylsulfonyl fluoride to the supernatants reduced protease activity to undetectable levels.

Physical characterization of the cloned DNA. A partial restriction map of the 6.5-kb *Eco*RI insert is shown in Fig. 1. The subtilisin gene was localized to within the 2.5-kb *Kpn*I-*Eco*RI fragment by subcloning in pBS42 the two *Kpn*I-*Eco*RI fragments and testing for overexpression of subtilisin in *B. subtilis* BG84. Southern hybridization experiments with a labeled fragment from the C terminus of the *B. amyloliquefaciens* subtilisin gene as a probe localized the C terminus of the *B. subtilis* gene to within or part of the 631-bp *Hin*cII fragment B in the right-end *Kpn*I-*Eco*RI fragment (Fig. 1). The tandem *Hin*cII fragments B, C, and D and *Hin*cII-*Eco*RI

fragment E (Fig. 1) were ligated into the appropriate M13 vectors for DNA sequence analysis. The sequence of this region is shown in Fig. 2.

Mapping of the subtilisin structural gene by PBS1 transduction. The 516-bp *Hin*cII fragment C, which contains part of the putative "pro" sequence and approximately 50% of the coding sequence for mature subtilisin (Fig. 1 and 2), was ligated into pJH101 that had been digested with *Hin*cII and treated with bacterial alkaline phosphatase. This plasmid, pIV6, was transformed into *B. subtilis* 1168 and strain BG31, and single colony transformants were colony purified twice and used to prepare PBS1 transducing lysates. These strains were designated BG2014 and BG2015, respectively (Table 1). Strains IA84 and IA151 were transduced with a PBS1 lysate made from BG2014, and chloramphenicol resistance was found to be linked to *glyB* (83%) and *argC* (60%). Additional linkage data were accumulated in a three-factor cross. (*glyB*, Cm^r, *metA*), when BG31 was transduced with this same lysate. The results of a five-factor cross with *glyB*, *catA*, *metD*, and *argC* are shown in Table 2. Figure 3 summarizes the linkage data and shows the order to be (*glyB catA*) Cm^r *metD argC metA*.

Isolation of the structural gene deletion mutation. To construct an integration plasmid carrying a defective subtilisin gene with a 684-bp deletion, a two-step ligation was required. These constructions are illustrated in Fig. 4. The plasmid carrying the 6.5-kb insert, pS168.1, was digested with *Eco*RI, and the single-stranded ends were filled in with the appropriate deoxynucleotides by treatment with the Klenow fragment of DNA polymerase I. This DNA was restricted with *Hin*cII, and the 800-bp *Eco*RI-*Hin*cII fragment E (Fig. 1), which contains, in part, the 5′ end of the subtilisin gene, was purified by electroelution from a preparative polyacrylamide gel. This fragment was ligated into pJH101 that had been digested with *Hin*cII. The resultant plasmid, pIDV1, contained fragment E in the orientation shown in Fig. 4. *Hin*cII fragment B of pS168.1, which contains the 3′ end of the subtilisin gene, was gel purified as described above and ligated into pIDV1 that had been digested at the unique *Hin*cII site. Restriction analysis of the resulting plasmids identified one, designated pIDV1.4, that contained fragment B in the correct orientation with respect to fragment E. This plasmid pIDV1.4 contains a deletion derivative of the subtilisin gene and portions of the 5′ and 3′ flanking sequences.

Upon transformation of *B. subtilis* BG77 (*prt-77*, Prt^{-r}) with pIDV1.4, two classes of Cm^r transformants were obtained, 75% showed parental level of proteases (Prt^{+r}), and 25% appeared almost completely protease deficient (Prt$^-$) as observed by zones of clearing on plates containing LB agar plus skim milk. The Cm^r Prt$^-$ class was unexpected and could not be due to a single crossover integration of the plasmid at the homologous regions for fragment E or B because the gene would not be interrupted. In fact, when either fragment E or B was ligated independently into pJH101 and subsequently transformed into *B. subtilis* BG77, the protease-deficient phenotype was not observed (data not shown). The Cm^r phenotype of Cm^r Prt$^-$ pIDV1.4 transformants was found to be unstable, and Cm^s Prt$^-$ derivatives could be isolated at a frequency of about 0.1% after 10 generations of growth in minimal medium in the absence of antibiotic selection.

Chromosomal DNA was obtained from BG77 and the Cm^s Prt$^-$, Cm^r Prt$^-$, and Cm^r Prt^{+r} derivatives of transformations with pIDV1.4. These chromosomal DNA preparations were digested with *Eco*RI and *Nco*I, electrophoresed on agarose gels, and transferred to nitrocellulose filters. The hybridization pattern of these DNAs, when probed with the labeled 2.5-kb *Kpn*I-*Eco*RI fragment (Fig. 1), is shown in Fig. 5. The structure of the chromosome at the wild-type subtilisin locus is diagramatically shown and confirmed by the hybridization data in lane b. The 1.7-kb *Eco*RI-*Nco*I band contains the 684-bp sequence to be deleted. Lane d is DNA isolated from a Cm^r Prt^{+r} transformant (BG2016). As seen from data, the structure of this particular Cm^r Prt^{+r} transformant was derived from a single crossover and integration of the plasmid at the homologous region for fragment E. Lane c contains DNA isolated from a Cm^r Prt$^-$ transformant (BG2017); when the structure is determined from the Southern data, the only difference between the Cm^r Prt$^-$ and Cm^r Prt^{+r} strains is the 684-bp deletion (fragments C and D) within the subtilisin structural gene. Lane a contains DNA from the Cm^s Prt$^-$ strain (BG2018) and is a Cm^s derivative of BG2017 (lane c). No plasmid DNA remained at the subtilisin locus, but, as expected, the *Eco*RI-*Nco*I fragment is reduced in size from 1.7 kb (lane b) to 1.0 kb (lane a). Other enzymatic digests and Southern blots have confirmed the architecture of these DNAs to be as illustrated in Fig. 5 (data not shown).

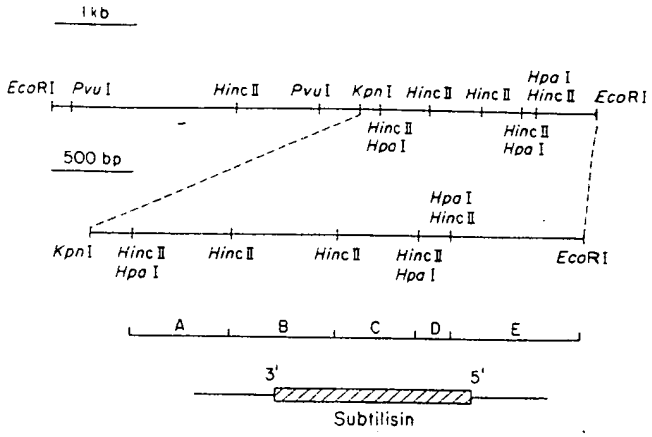

FIG. 1. Partial restriction map of the 6.5-kb *Eco*RI fragment that contains the functional *B. subtilis* 1168 subtilisin structural gene. A functional gene is contained within the expanded map of the 2.5-kb *Kpn*I-*Eco*RI subclone. The tandem *Hin*cII fragments and the *Hin*cII-*Eco*RI fragment are functionally labeled with the letters A through E to aid in the description of plasmid constructions and the data presented in Fig. 5 and 6. Fragments C and D are entirely within the proposed translated sequence of subtilisin, whereas fragments B and E contain part of the 3′ and 5′ translated sequence, respectively.

The genotypic designations for the pIDV1.4 transformants and derivatives were assigned as follows. The Cm^s Prt$^-$ phenotype carries no detectable plasmid DNA, but does carry a 684-bp deletion within the subtilisin structural gene (*Δapr-684*). The Cm^r Prt$^-$ phenotype carries the deletion and pIDV1.4 (*Δapr-684*::pIDV1.4), whereas the Cm^r Prt^{+r} strain has no mutation, but carries pIDV1.4 integrated at the *apr* locus (*apr*::pIDV1.4).

Because the exact nature of the *prt-77* mutation in *B. subtilis* BG77 was not fully understood and to simplify interpretations of the protease assay and sporulation data, the *Δapr-684* allele was transferred to *B. subtilis* IA84 by PBS1 transduction. The *apr* locus maps between *glyB* and *metD*, so GlyB$^+$ MetD$^+$ transductants were selected by infecting *B. subtilis* IA84 with a transducing lysate prepared

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   1 GATATACCTAAATAGAGATAAAATCATCTCAAAAAAATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAG

                                                          -100
                                  fMet Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu
 101 TCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGA GTG AGA AGC AAA AAA TTG TGG ATC AGC TTG TTG TTT GCG TTA ACG TTA

     -90                                -80                                         -70
     Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala Gly Lys Ser Ser Thr Glu Lys Lys Tyr Ile Val
 185 ATC TTT ACG ATG GCG TTC AGC AAC ATG TCT GCG CAG GCT GCC GGA AAA AGC AGT ACA GAA AAG AAA TAC ATT GTC

                     -60                                 -50
     Gly Phe Lys Gln Thr Met Ser Ala Met Ser Ser Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val
 260 GGA TTT AAA CAG ACA ATG AGT GCC ATG AGT TCC GCC AAG AAA AAG GAT GTT ATT TCT GAA AAA GGC GGA AAG GTT

     -40                                -30                                         -20
     Gln Lys Gln Phe Lys Tyr Val Asn Ala Ala Ala Ala Thr Leu Asp Glu Lys Ala Val Lys Glu Leu Lys Lys Asp
 335 CAA AAG CAA TTT AAG TAT GTT AAC GCG GCC GCA GCA ACA TTG GAT GAA AAA GCT GTA AAA GAA TTG AAA AAA GAT

                     -10                                     -1   1                                   10
     Pro Ser Val Ala Tyr Val Glu Glu Asp His Ile Ala His Glu Tyr Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln
 410 CCG AGC GTT GCA TAT GTG GAA GAA GAT CAT ATT GCA CAT GAA TAT GCG CAA TCT GTT CCT TAT GGC ATT TCT CAA

                                         20                                     30      32
     Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
 485 ATT AAA GCG CCG GCT CTT CAC TCT CAA GGC TAC ACA GGC TCT AAC GTA AAA GTA GCT GTT ATC GAC AGC GGA ATT

                         40                                      50                                   60
     Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp
 560 GAC TCT TCT CAT CCT GAC TTA AAC GTC AGA GGC GGA GCA AGC TTC GTA CCT TCT GAA ACA AAC CCA TAC CAG GAC

                 64                      70                                      80
     Gly Ser Ser His Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ser
 635 GGC AGT TCT CAC GGT ACG CAT GTA GCC GGT ACG ATT GCC GCT CTT AAT AAC TCA ATC GGT GTT CTG GGC GTT AGC

                         90                                     100                                  110
     Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly
 710 CCA AGC GCA TCA TTA TAT GCA GTA AAA GTG CTT GAT TCA ACA GGA AGC GGC CAA TAT AGC TGG ATT ATT AAC GGC

                                        120                                     130
     Ile Glu Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Thr Gly Ser Thr Ala Leu
 785 ATT GAG TGG GCC ATT TCC AAC AAT ATG GAT GTT ATC AAC ATG AGC CTT GGC GGA CCT ACT GGT TCT ACA GCG CTG

                        140                                     150                                  160
     Lys Thr Val Val Asp Lys Ala Val Ser Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
 860 AAA ACA GTC GTT GAC AAA GCC GTT TCC AGC GGT ATC GTC GTT GCT GCC GCA GCC GGA AAC GAA GGT TCA TCC GGA

                                        170                                     180
     Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala Val Gly Ala Val Asn Ser Ser Asn Gln
 935 AGC ACA AGC ACA GTC GGC TAC CCT GCA AAA TAT CCT TCT ACT ATT GCA GTA GGT GCG GTA AAC AGC AGC AAC CAA

                        190                                     200                                  210
     Arg Ala Ser Phe Ser Ser Ala Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro
1010 AGA GCT TCA TTC TCC AGC GCA GGT TCT GAG CTT GAT GTG ATG GCT CCT GGC GTG TCC ATC CAA AGC ACA CTT CCT

                                        220 221                                 230
     Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu
1085 GGA GGC ACT TAC GGC GCT TAT AAC GGA ACG TCC ATG GCG ACT CCT CAC GTT GCC GGA GCA GCA GCG TTA ATT CTT

                        240                                     250                                  260
     Ser Lys His Pro Thr Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr Leu Gly Asn Ser
1160 TCT AAG CAC CCG ACT TGG ACA AAC GCG CAA GTC CGT GAT CGT TTA GAA AGC ACT GCA ACA TAT CTT GGA AAC TCT

                                        270
     Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala Ala Ala Gln OC
1235 TTC TAC TAT GGA AAA GGG TTA ATC AAC GTA CAA GCA GCT GCA CAA TAA  TAGTAAAAAGAAGCAGGTTCCTCCATACCTGCTTC

1318 TTTTTATTTGTCAGCATCCTGATGTTCCGGCGCATTCTCTTCTTTCTCCGCATGTTGAATCCGTTCCATGATCGACGGATGGCTGCCTCTGAAAATCTTC

1418 ACAAGCACCGGAGGATCAACCTGCTCAGCCCCGTCACGGCCAAATCCTGAAACGTTTTAACACTGGCTTCTCTGTTCTCTGTC
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FIG. 2. Nucleotide sequence of the subtilisin structural gene and the adjacent regions. The inferred translated amino acid sequence is also shown. The putative ribosome binding sites and transcriptional terminator are the underlined sequences. The *Δapr-684* allele is characterized as a 684-bp deletion mutation beginning with nucleotide 178 and ending with nucleotide 871.

from BG2018. DNA was prepared from several transductants, and introduction of the deletion mutation was confirmed by Southern hybridization experiments (data not shown). When supernatants of *B. subtilis* carrying *Δapr-684* (BG2019) and the wild-type allele (IA84) were assayed for extracellular serine protease activity, BG2019 produced 10-fold less than IA84. This difference was apparent at the 4-, 6-, and 8-h time points in the stationary phase of growth in modified Schaeffer's medium. In addition, strains carrying either the mutant or the wild-type allele were found to produce 80 to 100% heat-resistant endospores after 24 h of growth in modified Schaeffer medium.

TABLE 2. Analysis of the *glyB33*, *catA*, *apr-1*::pIV6, *metD1*, and *argC4* determinants[a]

Phenotype (no. scored)	% of recombinants in the following class[b]											
	A	B	C	D	E	F	G	H	I	J	K	L
Cm^r (85^+)	47.1	18.4	11.6	11.6		4.6	3.4	1.1	1.1	1.1		
Gly^+ (60)	36.7	13.3	18.3		26.7						3.3	1.1

[a] Strain IA84 (Gly^- Cat^- Cm^s Met^- Arg^+) was transduced with a PBS1 lysate made from strain BG2021 (Gly^- Cat^- Cm^r Met^- Arg^+). See Table 1 for genotypes.

[b] Classes: A, Gly^- Cat^- Cm^r Met^- Arg^+; B, Gly^+ Cat^- Cm^r Met^- Arg^+; C, Gly^- Cat^- Cm^r Met^- Arg^+; D, Gly^- Cat^- Cm^r Met^- Arg^+; E, Gly^- Cat^- Cm^s Met^- Arg^+; F, Gly^- Cat^+ Cm^r Met^- Arg^+; G, Gly^- Cat^- Cm^r Met^+ Arg^+; H, Gly^- Cat^- Cm^r Met^- Arg^+; I, Gly^- Cat^- Cm^r Met^- Arg^-; J, Gly^- Cat^+ Cm^r Met^- Arg^+; K, Gly^+ Cat^- Cm^s Met^- Arg^+; L, Gly^+ Cat^- Cm^s Met^- Arg^-.

DISCUSSION

These data show that the cloned gene is the subtilisin structural gene of *B. subtilis* I168. When this gene was expressed on a high-copy-number plasmid in I168, a fivefold increase in phenylmethylsulfonyl fluoride-sensitive protease activity was observed. The DNA sequence of the gene is 80% homologous to the *B. amyloliquefaciens* subtilisin structural gene (46), and the translated mature coding sequence is 85% homologous to the protein sequence of subtilisin BPN' (21). Furthermore, the subtilisin amino acid active site residues, asp-32, his-64, and ser-221 are conserved in the translated sequence (21). Strongin et al. (42) reported the purification and partial N-terminal amino acid sequence of a *B. subtilis* intracellular serine protease. This protein was only 50% homologous to the N termini of the subtilisins; thus it is unlikely that the cloned gene codes for that intracellular protease. In fact, southern hybridization experiments at low-stringency conditions with the *B. subtilis* subtilisin gene as a probe showed no evidence for a second homologous gene (data not shown).

When an in vitro-derived deletion mutation was introduced into the chromosome of strain BG77, a phenotypic decrease in protease activity was detected on LB plus skim milk agar plates. Supernatants from cultures carrying the subtilisin deletion mutation (BG2019) produced only 10% of wild-type phenylmethylsulfonyl fluoride-sensitive protease levels. A certain level of background protease activity was expected because of cell lysis and release of intracellular serine proteases during the growth of the cultures and because of the secreted esterase (bacillopeptidase F), which has been recently described as a serine protease (33). It was difficult to distinguish between the IA84 wild-type phenotype on LB plus skim milk agar and the phenotype of BG2019, which carries the *Δapr-684* allele. This perhaps is one of the reasons why the isolation of mutations in the structural gene of subtilisin has eluded most investigators to date. However, if the *Δapr-684* allele was introduced into a strain carrying the *prt-77* mutation, the difference between the Apr^- and Apr^+ phenotypes on skim agar was clear. We suspect that the *prt-77* mutation is in the structural gene of the neutral protease, but our analysis is incomplete, and confirmation awaits additional data.

As shown in Fig. 2, assignments were made for a putative Shine-Dalgarno sequence (38) and translational start for the *B. subtilis* I168 subtilisin gene. The necessity for strong complementarity ($\Delta G < -11$ kcal [ca. -46.1 kJ]) between the Shine-Dalgarno sequence of the mRNA and the 3'-OH end of the 16S rRNA for efficient translation in *Bacillus* spp. and *Staphylococcus* spp. genes has been postulated (23, 24, 29). Two possible Shine-Dalgarno sequences were identified within 350 bp of the start of the mature coding sequence and are shown in Fig. 2 as underlined sequences. One sequence (AAAGGCGG) is 129 bp from the start of the mature coding sequence, and the other (AAAGGAGAG) is 330 bp from the start. As calculated by the method of Tinoco et al. (43), the free energies of formation of the most stable double helical pairing between the putative Shine-Dalgarno sequences and the 3'-OH end of rRNA were -11.6 kcal (ca. -48.6 kJ) and -15.4 kcal (ca. -64.5 kJ), respectively. Only the sequence AAAGGAGAG has a translational start codon (GUG) reasonably spaced (9 bp) 3' and in frame with the mature coding sequence. The use of GUG as a translational start codon in procaryotic genes has been reported (11).

It has been proposed (46) that the *B. amyloliquefaciens* subtilisin message is translated as a "prepro" polypeptide. This seems to also be the case for the *B. subtilis* I168 subtilisin. In addition to the 275-amino-acid mature coding sequence, the putative translated polypeptide includes 106 additional amino acids of unknown function. The first 23 amino acids make up a classic signal peptide that includes three positively charged residues within the first 5 amino acids. The following 12 amino acid residues appear to be the hydrophobic core of a signal peptide. A putative signal peptidase site is six residues from the end of the hydrophobic core and follows the tripeptide Ala-Phe-Ser. The signal peptide and signal processing site predictions are based on the consensus data of Perlman and Halvorson (30). The approximately 83 amino acids between the signal sequence and the mature coding sequence is the putative pro sequence; it is unlikely this sequence acts as a signal peptide because 33% of its residues are charged amino acids (69% of these are positively charged), and no reasonable stretch of amino acids resembles a hydrophobic core.

The work of Anderson et al. (3), comparing the nucleotide sequences of human and bovine mitochondrial DNA, provides further evidence that the putative prepro is translated. When homologous translated sequences were examined, they found the least amount of variability in codon position 2, followed by positions 1 and then 3. This relationship deteriorated when untranslated sequences were compared. The *B. amyloliquefaciens* and *B. subtilis* I168 subtilisin sequences were compared for codon position variability; the prepro sequences showed least variability in codon position

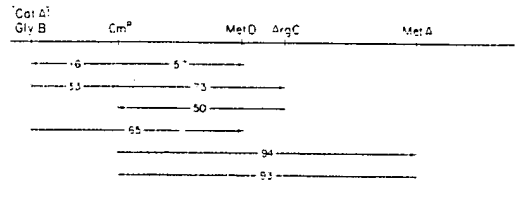

FIG. 3. Linkage relationships among the *catA*, *glyB33*, *apr-1*::pIV6 (Cm^r), *metD1*, *argC4*, and *metA29* markers. Numerical values (100 minus the estimated cotransductional frequencies) are the averages of at least two separate experiments. The arrows point from the selected to the unselected markers.

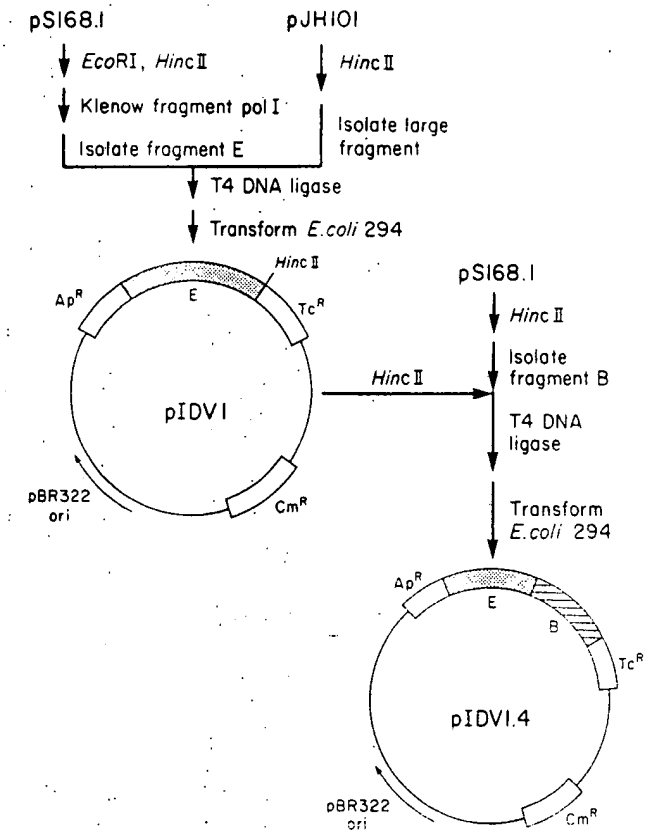

FIG. 4. Construction of the subtilisin deletion plasmid pIDV1.4. Fragments E and B, when ligated in tandem as they appear in Fig. 1, consist of the subtilisin gene with a 684-bp deletion within the translated coding sequence. Plasmid pIDV1.4 contains no origin of replication that functions in *B. subtilis*; when transformed into *B. subtilis*, it integrates via recombination between homologous sequences on the plasmid and the chromosome.

2 (11%), followed by positions 1 (22%) and then 3 (67%). Examination of the mature subtilisin sequences revealed similar numbers (position 2, 12%; position 1, 19%; position 3, 69%).

A promoter sequence for the recognition of RNA polymerase and initiation of transcription could not be identified by homology comparisons with consensus promoter sequences recently described by Johnson et al. (16). However, since the expression of subtilisin is closely associated with the onset of sporulation and many mutations blocking sporulation at early stages affect expression levels of subtilisin (14, 31), transcription of the subtilisin gene is probably under the control of a developmentally regulated promoter. An inverted and repeated sequence ($\Delta G = -22.2$ kcal [ca. 93.0 kJ]; 42) located in the 3′ flanking end of the gene (Fig. 2) may be a transcriptional terminator because similar sequences can be found in termination regions of *E. coli* genes (34) and *Bacillus* spp. genes (37, 46, 47).

The expected pathway for replacement of a chromosomal segment with an altered sequence in *S. cerevisiae* and *E. coli* has been well defined (12, 36, 39). In the case of subtilisin, integration of pIDV1.4 is mediated by a single crossover between fragment E or B and their homologous regions in the chromosome. In either case, both fragments E and B are directly duplicated in the resulting structure. Resolution of the integrated plasmid by a single crossover between one of the two pairs of direct repeats will result either in the restoration of the wild-type gene or a 684-bp deletion within the subtilisin structural gene. Consequently, chloramphenicol-sensitive derivatives of the strain carrying the integrated plasmid must be screened for the mutant phenotype.

An unexpected class of transformants was discovered when competent cells of BG77 were transformed with pIDV1.4. Twenty-five percent of the Cm^r transformants had a protease-deficient phenotype. This class of transformants could not be due to a single crossover integration of pIDV1.4 at either of the homologous regions because the subtilisin gene would not be interrupted. We reasoned that the Cm^r Prt^- phenotype was derived from a double crossover replacement between the homologous chromosomal region and a concatemer of pIDV1.4 (Fig. 5 and 6). This hypothesis seems reasonable since linear plasmid concatemers have been proposed as intermediates in the pathway for plasmid transformation in *B. subtilis* (8). At a minimum, a dimer of pIDV1.4 is required for this replacement pathway. Resolution of this structure by a single crossover between either of the two directly repeated sequences results in the excision of the plasmid DNA and the retention of the deletion within the subtilisin structural gene (Fig. 5 and 6).

In summary, a variation of a technique termed gene replacement was adapted for use in *B. subtilis* 1168 to introduce a 684-bp deletion within the subtilisin structural gene. This technique is useful in that defined deletion

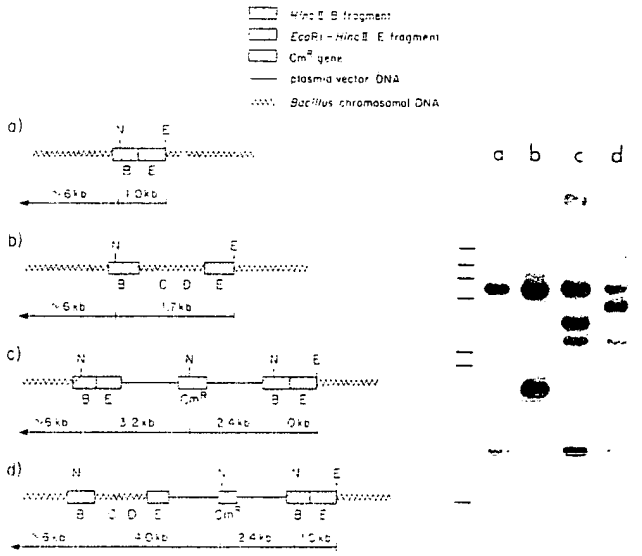

FIG. 5. Structural analysis of plasmid pIDV1.4 integrates and derivatives. Shown are Southern hybridization data and the corresponding proposed structures of wild-type DNA (Cm^s Prt^+, BG77, lane b), DNA from strains carrying the *Δapr-684*::pIDV1.4 allele (Cm^r Prt^-, BG2017, lane c), the *Δapr-684* allele (Cm^s Prt^+, BG2018, lane a), and the *apr*::pIDV1.4 allele (Cm^r Prt^-, BG2016, lane d). The Southern blot shown is composed of *EcoRI-NcoI* digests of these DNAs hybridized with the labeled 2.5-kb *KpnI-EcoRI* fragment. The *EcoRI* sites are designated E, and the *NcoI* sites are designated N. The proposed structures show the size and number of bands expected for each of these DNAs. Other digestions and Southern hybridization experiments (data not shown) have confirmed these structures. The horizontal lines next to the Southern blots correspond to fragments 1 through 7 of *Hin*dIII-digested λ DNA, with sizes of 23.1, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 kb, respectively.

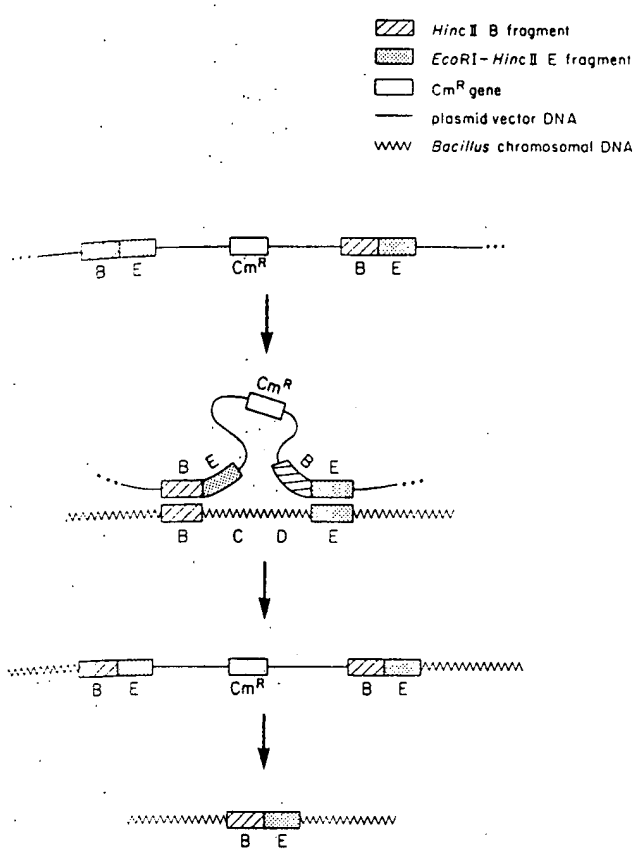

FIG. 6. Possible pathway of deletion formation in *B. subtilis*. A concatemer of plasmid pIDV1.4 interacts via fragments B and E with their homologous regions at the chromosomal subtilisin locus. A double crossover replacement of the wild-type gene with pIDV1.4 and flanking copies of both fragment E and B occurs. As a result of this, fragments C and D are deleted, and fragments E and B are directly duplicated. This structure was manifested phenotypically (Cm^r Prt$^-$) and confirmed by Southern hybridization experiments (Fig. 5, lane c). Resolution of this structure by a homologous recombination event between the duplicated fragments B or E results in the loss of the plasmid sequence and maintenance of the 684-bp deletion (Fig. 5, lane a).

mutations of cloned genes can be introduced into the chromosome and easily detected by directly screening the phenotypes of plasmid transformants for the desired gene deletion. The plasmid phenotype is unstable because of flanking directly repeated DNA and is lost at a frequency of about 0.1% after about 10 generations of growth in nonselective media, but the deletion mutation is retained. It is apparent from this study that the subtilisin gene product is not required for normal sporulation in *B. subtilis* I168.

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ADDENDUM IN PROOF

Since this paper was submitted, Wong et al. (46a) have reported the DNA sequence of a fragment of the *B. subtilis* subtilisin gene and its map location in the *B. subtilis* chromosome. The results reported by Wong et al. are in agreement with the results we obtained.

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Construction of a highly efficient host-vector system for secretion of heterologous protein in *Bacillus subtilis*

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Summary

An efficient secretion host-vector system in *Bacillus subtilis* was constructed using two *Bacillus amyloliquefaciens* genes, *sacQ* (*BamF*) responsible for the increase of extracellular levansucrase and protease levels and *npr* for extracellular neutral protease. The former was introduced into the genomic DNA of the extracellular alkaline and neutral protease deficient *Bacillus subtilis* and the latter was used to construct a secretion vector utilizing the promoter and head portion of the prepropeptide coding region. This introduction of *sacQ*(*BamF*) permitted the increase of levansucrase level of the recipient strain. In contrast, the residual proteolytic activity of the double mutant was further reduced by *sacQ*(*BamF*). The expression level of chloramphenicol acetyltransferase gene located in a multicopy plasmid in the double mutant, of which mRNA synthesis was directed by the *npr* promoter, was elevated 1.7-fold by the introduction of *sacQ*(*BamF*). The extracellular production level of human growth hormone (hGH) in the double mutant, whose gene was inserted into the multicopy secretion vector based on *npr*, was increased 4- to 5-fold by the introduction of *sacQ*(*BamF*). This increase seemed to be mainly dependent upon the continuity of the hGH accumulation even in long cultivation rather than stimulation of the production rate. Using this host-vector system, we demonstrated the highly efficient secretion of hGH (200 mg l^{-1}) in *B. subtilis*.

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Extracellular enzyme overproducing gene; Host construction; Neutral protease gene; Secretion vector; Human growth hormone; *Bacillus subtilis*

Introduction

Recent advances in recombinant DNA technique have made secretion of the heterologous protein in *Bacillus subtilis* possible (Palva et al., 1983; Yamane et al., 1985; Honjo et al., 1985). However, the production level of the mammalian protein is still low mainly due to the proteolytic activity of the host *B. subtilis* (Ulmanen et al., 1985). Even in the extracellular alkaline and neutral protease deficient *B. subtilis*, the accumulation level of human interferon-β (Honjo et al., 1986b) or human growth hormone (hGH) (Honjo, unpublished data) was reduced by the residual proteolytic activity. Therefore to increase the extracellular production level of mammalian protein in *B. subtilis*, further reduction of the proteolytic activity present is necessary even in the double protease mutant.

On the other hand, enhancement of the expression of the heterologous gene is considered to be contributory to the increased accumulation of the target product. Previously, we have reported the cloning, sequencing and expression of a novel *B. amyloliquefaciens* gene involved in the increase of extracellular protease levels (Tomioka et al., 1985). Since Yang et al. (1986) have isolated the counterpart of this gene from *B. subtilis* and proved it to be *sacQ* by mapping, the *B. amyloliquefaciens* gene is denoted *sacQ(BamF)* here. In the $sacQ^h$ strain of *B. subtilis*, the activity levels of levansucrase and proteases are increased (Kunst et al., 1974). Shimotsu and Henner (1986) have shown that the elevated levansucrase level in the $sacQ^h$ mutant, results from the constitutive increase of its transcripts. This suggests that the transcripts of proteases also may be increased by *sacQ(BamF)* in *B. subtilis*.

It is interesting, therefore, to investigate whether *sacQ(BamF)* stimulates the expression of a heterologous gene of which transcription is directed by the *B. amyloliquefaciens npr* promoter in *B. subtilis*. However, the introduction of *sacQ(BamF)* into a wild-type strain on proteases of *B. subtilis* caused the increase of extracellular protease levels (Tomioka et al., 1985). It is considered, therefore, essential to use extracellular protease deficient strain as a recipient of *sacQ(BamF)* to avoid degradation of the target product by increased proteolytic activity. In this way it is interesting to discover whether the *sacQ(BamF)* introduced into an extracellular alkaline and neutral protease deficient strain can stimulate the residual proteolytic activity or not.

In this paper, we describe the introduction of *sacQ(BamF)* into a double extracellular protease deficient (*apr npr*) strain of *B. subtilis* by genetic recombination and the characteristics of the resultant strain. The expression of heterologous gene by the *npr* promoter in the obtained strain is also reported. Furthermore, highly efficient production of hGH using the *npr*-based secretion vector and the resultant strain is discussed.

Materials and Methods

Bacterial strain, media and transformation

B. subtilis MT400, a derivative of Marburg 168, is a genetically engineered protease deficient strain of which relevant genotype is *npr npr rec*$^{+}$. Unless noted otherwise, 2 × Luria broth (LB) containing 0.2% glucose was used as liquid medium. To detect halos around colonies, a casein plate was used (Honjo et al., 1984). Protoplast transformation was carried out according to Chang and Cohen (1979). Competent cell transformation was performed as described by Saito et al. (1961). A plasmid pPL603 was prepared from *B. subtilis* 1E31 (Bacillus Genetic Stock Center, Ohio University).

Introduction of sacQ(BamF) into MT400 genomic DNA

The plasmid pNP181 (1 μg) was added to 500 μl of competent culture of MT400. After the plasmid addition, the culture was incubated for 1 h at 37° C. Then one ml of LB supplemented with 7.5 mg ml^{-1} of kanamycin was added to the culture. After 12 h cultivation at 37° C, cells were harvested by centrifugation. The cells were then resuspended in Schaeffer sporulation medium and cultured with vigorous shaking for 24 h at 37° C. An aliquot of the culture was diluted 10^{5}-fold with saline and heated at 80° C for 15 min then plated onto TBAB-plates containing no antibiotics. Colonies grown on the plates were transferred to TBAB-kanamycin (5 μg ml^{-1}) plates and casein plates to check kanamycin resistancy and caseinolytic activity, respectively. The loss of the plasmid in the kanamycin sensitive transformant, of which caseinolytic activity was repressed, was confirmed by alkaline plasmid preparation method (Birnboim and Doly, 1979). Morphological change of the transformant was examined by phase-contrast microscopy.

SDS-polyacrylamide gel electrophoresis and Western analysis

SDS-polyacrylamide gel electrophoresis (PAGE) was done according to Laemmli (1970). Western analysis was carried out using Bio-Rad Immun-Blot (GAR-HRP) according to supplier's manual. Rabbit antiserum against human pituitary GH was generously supplied by K. Nakajima.

Assays of enzymes

Extracellular protease activity was measured by caseinolysis as described previously (Honjo et al., 1984) except for the reaction condition. Here, the reaction was carried out for 6 h at 40° C. Levansucrase activity was assayed by the method of Dedonder (1966). Chloramphenicol acetyltransferase (CAT) activity was determined colorimetrically using cleared cell lysate according to Shaw (1975) and is expressed as μmol of acetylated chloramphenicol per min at 37° C per mg of protein.

Assays of protein and hGH

Protein concentration was assayed by the method of Lowry et al. (1951). Total **extracellular protein** was determined using the precipitates obtained by trichloro-**acetic acid addition** to the culture supernatant. The concentration of hGH was **measured by solid phase** enzyme immunoassay as described previously (Honjo et al., **1986a).**

Results

Introduction of sacQ(BamF) into genomic DNA of MT400

We have previously cloned *sacQ(BamF)* in a plasmid pNP181 (Tomioka et al., 1985), whose structure is shown in Fig. 1. To examine the effect of *sacQ(BamF)* in

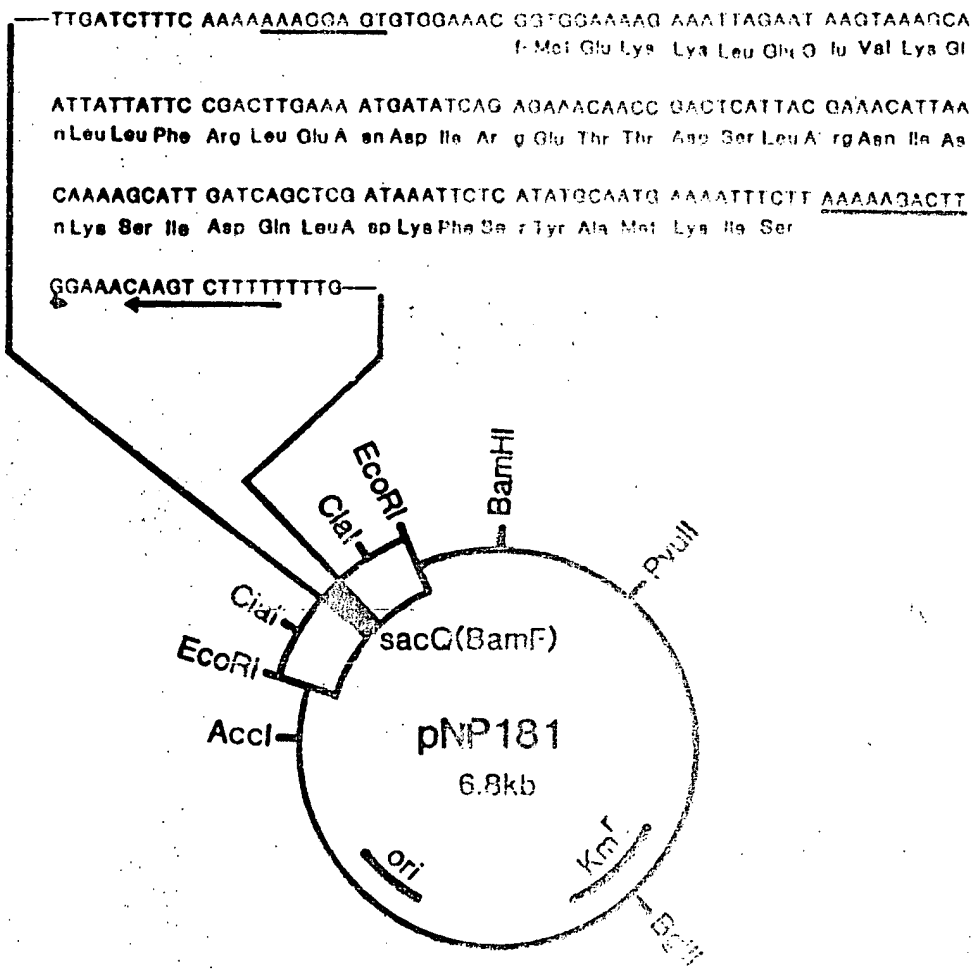

Fig. 1. Structure of *sacQ(BamF)* cloned in pNP181. The potential ribosome binding site is underlined. A sequence for tentative transcription terminator is shown by arrows.

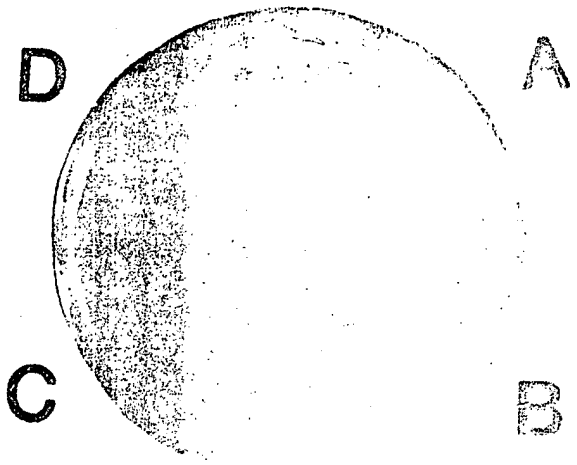

Fig. 2. Halo formation on a casein plate. A, 207-21(*aprA*$^+$ *nprE*$^+$) which is a parent strain of MT400; B, MT400; C, MT400(pNP181); D, MT430. These strains were inoculated onto a casein plate and incubated at 37°C for 60 h.

the double protease mutant, this plasmid was introduced into *B. subtilis* MT400, which forms a slight halo on a casein plate for long-time incubation (2 days), by protoplast transformation. The transformant obtained, MT400(pNP181), was observed to form no halo around the colony even after 60 h incubation at 37°C. Since this suggested that *sacQ*(*BamF*) did not stimulate the residual proteolytic activity of the double protease mutant, this gene was introduced into the genomic DNA of MT400 by competent cell transformation as described in Materials and Methods.

The strain obtained, designated MT430, bearing no plasmid also lost the halo-forming ability similarly to MT400(pNP181) (Fig. 2). To confirm the presence of *sacQ*(*BamF*) in the MT430 genomic DNA, we measured the levansucrase activity in the medium. The result (Table 1) showed that the enzyme level of MT430 is 15-fold higher than that of MT400. As for morphology, MT430 was filamentous similarly to MT400(pNP181) during logarithmic phase whereas MT400 was not (Fig. 3). Furthermore, we confirmed that the genomic DNA of MT430 could transform a *B. subtilis* wild-type strain on protease to be a protease overproducing strain. These results showed that MT430 bearing *sacQ*(*BamF*) in the genomic DNA.

TABLE 1

LEVANSUCRASE ACTIVITY

Cells were grown in LB medium supplemented with 2% sucrose at 37°C for 13 h. The activity was assayed using culture supernatant.

Strain	Growth (A660)	Relative activity
MT400	2.25	1.0
MT400(pNP181)	2.00	31.8
MT430	2.21	15.0

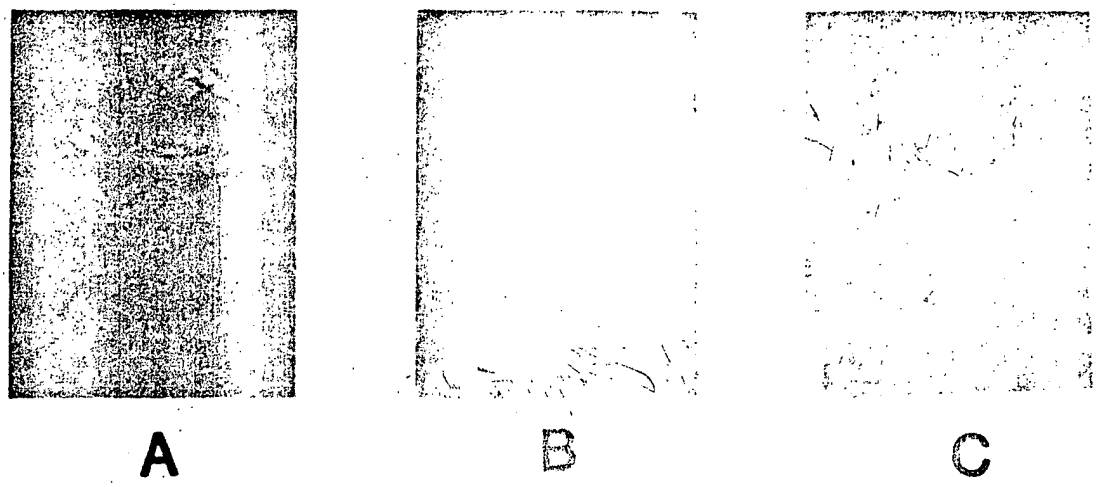

Fig. 3. Morphological change of MT400 by introducing *sacQ(BamF)*. A, MT400; B, MT400(pNP181); C, MT430. Cells were grown in Antibiotic Medium 3 (Difco Laboratories) at 37 °C for 3 h.

To examine the change of the extracellular protease level by the introduction of *sacQ(BamF)*, we measured the caseinolytic activities present in the media of MT430, MT400(pNP181) and MT400, which were cultured for 18 h at 30 °C. The relative activities in MT430 and MT400(pNP181) were 0.4 and 0.3, respectively, whereas that in MT400 was set at 1.0. As for growth, there was no difference among these three strains.

Expression of CAT gene by npr promoter in MT430

It has been reported that the expression of β-galactosidase gene of which transcription is directed by levansucrase gene (*sacB*) is enhanced in the $sacQ^h$ strain of *B. subtilis* (Shimotsu and Henner, 1986). Since protease levels are also elevated in the $sacQ^h$ strain, *sacQ(BamF)* might permit the increased expression of heterologous gene of which transcription was under the *B. amyloliquefaciens* protease-gene promoter in *B. subtilis*. To examine this, a plasmid pPN150-30 (Fig. 4), a derivative of pPL603 (Williams et al., 1981), containing a head portion of *B. amyloliquefaciens npr* preceding a CAT gene (*cat-86*), was introduced into MT430 and MT400. Then, CAT activities of both transformants were assayed. The result (Table 2) showed that the CAT activity of MT430 was 1.7-fold higher than that of MT400. Although the stimulation level was not so high, *sacQ(BamF)* could enhance the *npr*-directed expression of heterologous gene in *B. subtilis*.

Construction of the hybrid plasmid for hGH secretion

A plasmid pNP150 is a *B. amyloliquefaciens npr* clone which we constructed previously (Honjo et al., 1984; Shimada et al., 1985). Using this plasmid, a plasmid phGH928 containing a mature hGH gene following the *npr* segment for the promoter and head portion of the prepropeptide composed of 48 amino acid

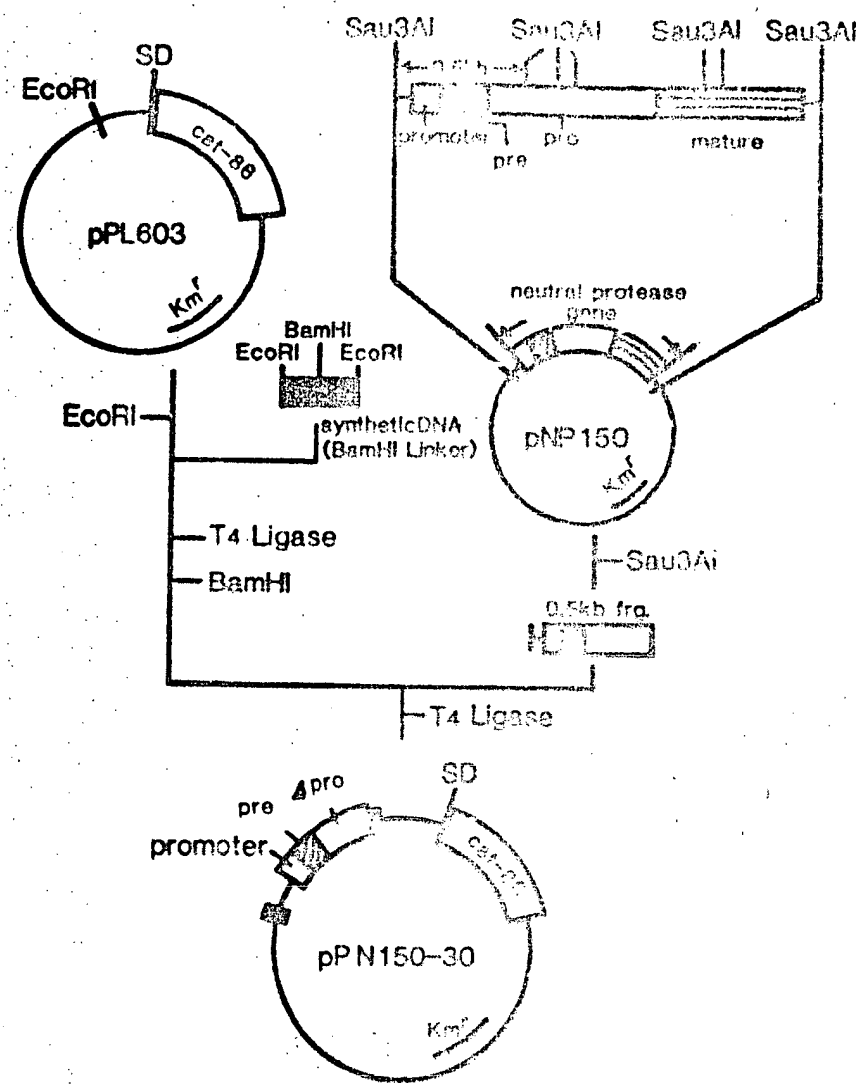

Fig. 4. Construction of pPN150-30 of which *cat* expression is under the control of *B. amyloliquefaciens npr* promoter.

residues was constructed (Fig. 5). The details of the construction and the secreted hGH derived from this plasmid have been described previously (Nakayama et al., 1987). Since phGH928 contains a mature *B. subtilis* α-amylase gene following the

TABLE 2

EXPRESSION OF *CAT-86* BY *B. amyloliquefaciens npr* PROMOTER

Cells were grown in 2×LB medium containing 25 μg ml^{-1} chloramphenicol and 0.2% glucose at 30 °C for 18 h.

Strain	CAT activity (Unit)
MT400(pPN150-30)	1350
MT430(pPN150-30)	2303

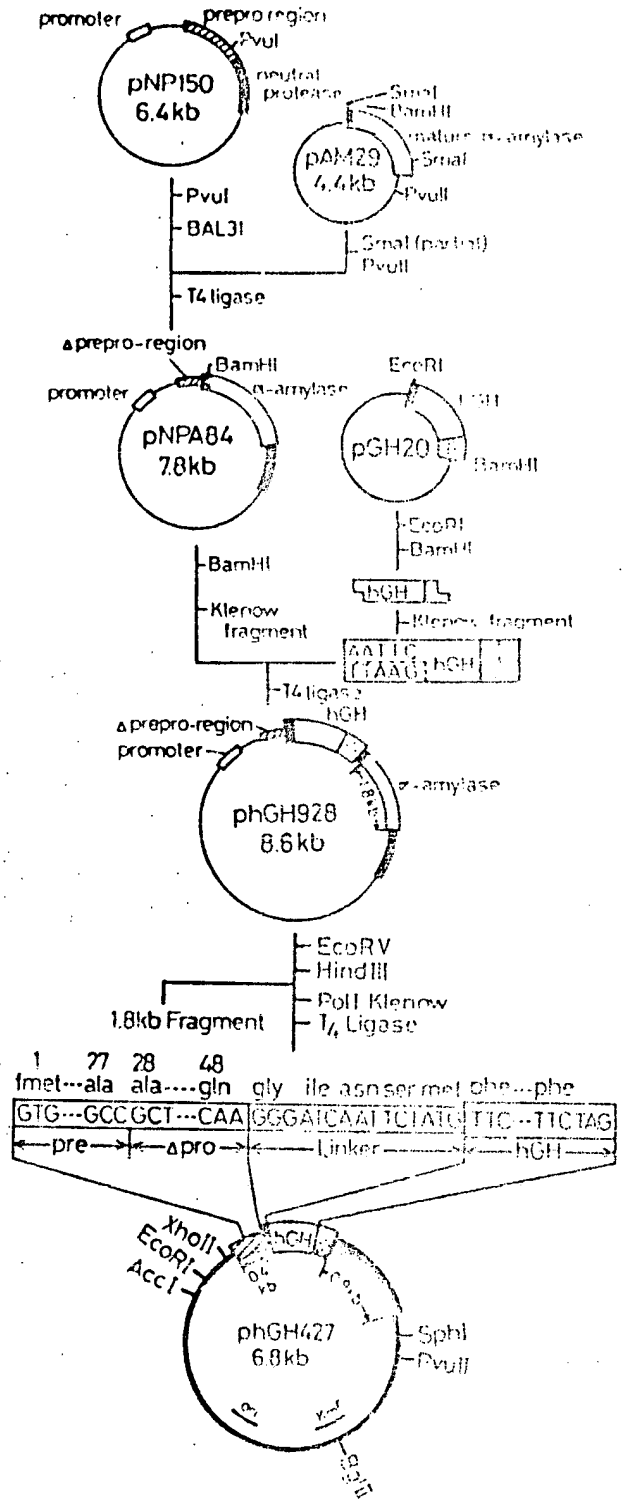

Fig. 5. Construction of phGH427 from pNP150.

hGH gene, which had been used as a probe to select an effective portion of *npr* for efficient secretion, we removed the α-amylase gene by the digestion with *Eco*RV and *Hin*dIII. Then the resultant protruding end was blunted using Klenow fragment and ligated (Fig. 5). The smaller plasmid obtained was designated phGH427, of which stability in long culture was improved by this removal of α-amylase gene (data not shown).

Secretion of hGH

The plasmid phGH427 was introduced into *B. subtilis* MT430 and MT400 by protoplast transformation. Both transformants were cultured for 15 h at 30 °C and the accumulated hGH in each medium was measured. The strain MT430 harboring phGH427 secreted 50 mg l^{-1} hGH whereas MT400(phGH427) secreted 11 mg l^{-1}. As for growth, the cell density was the same in both cultures (A660, 8.5). Since this indicated that the hGH productivity in MT430(phGH427) was improved 4.5-fold, we examined the feature of hGH secretion from the transformant using a 7-l reactor. As shown in Fig. 6 the hGH secretion was initiated at the late exponential or early stationary phase and increased up to 25 h, when the accumulation level of hGH reached 80 mg l^{-1}. The secreted hGH was one of the major proteins present in the culture supernatant (Fig. 7A) and the ratio to the total extracellular protein reached 17% in a 25-h culture (Fig. 6). This culture supernatant was subjected to double immunodiffusion test without any concentration of hGH. The result showed that the secreted hGH was indistinguishable from the pituitary derived hGH (data

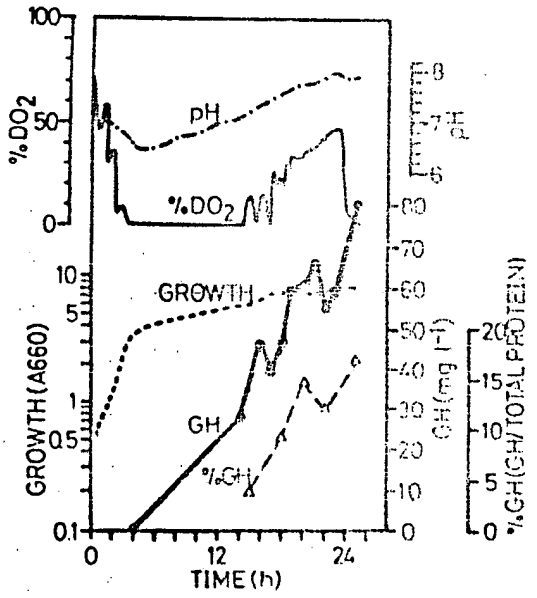

Fig. 6. Secretion of hGH from MT430(phGH427) using a 7-l bioreactor. Cells were grown in 4000 ml of medium at 30 °C. An aliquot of the culture was withdrawn at indicated times and cells were removed by centrifugation. The obtained culture supernatant was used for assays of hGH and total extracellular protein. Percent GH means the % ratio of hGH secreted to the total extracellular proteins.

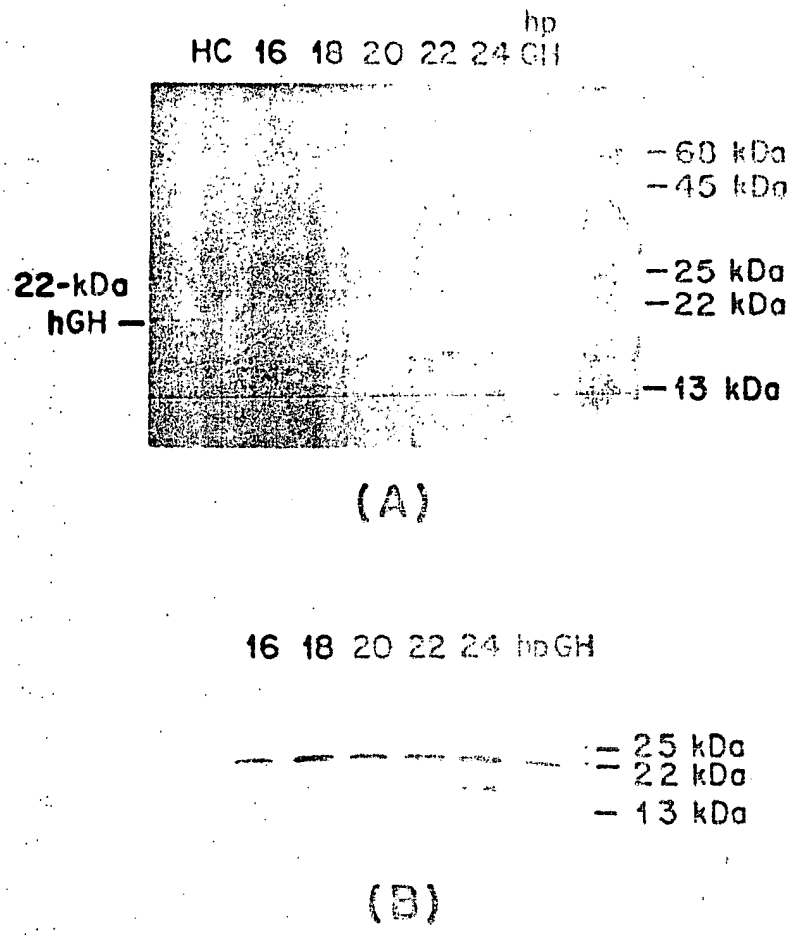

Fig. 7. SDS-polyacrylamide gel electrophoresis (A) and Western analysis (B) using culture supernatant of MT430(phGH427). Samples used were the same as those described in the legend to Fig. 6. As a control, MT430 harboring no plasmid was cultured similarly for 20 h. The precipitate obtained from the addition of trichloroacetic acid to 100 μl of the supernatant was dissolved in 35 μl of sample buffer containing 5% 2-mercaptoethanol. The resultant solution was heated at 100°C for 3 min and applied to SDS-PAGE. HC, host control; hpGH, pituitary derived hGH (2 μg).

not shown). Western analysis revealed that the majority of the secreted hGH was the same as authentic hGH in size (22 kDa) and that cleaved 15-kDa hGH was also present (Fig. 7B).

Secretion of hGH by medium exchange method

Recently, we have found that the accumulation of the heterologous protein in the culture medium of *B. subtilis* was increased by transferring the cells to fresh medium after growth (Honjo et al., 1986a). To confirm whether the medium exchange was suitable for this case, the strain MT430(phGH427) was cultured and the grown cells were transferred to the same volume of fresh medium. As shown in Fig. 8, the accumulation of hGH in the medium reached 210 mg l^{-1}. At this level, the ratio of the secreted hGH to the total extracellular proteins reached 30%. In

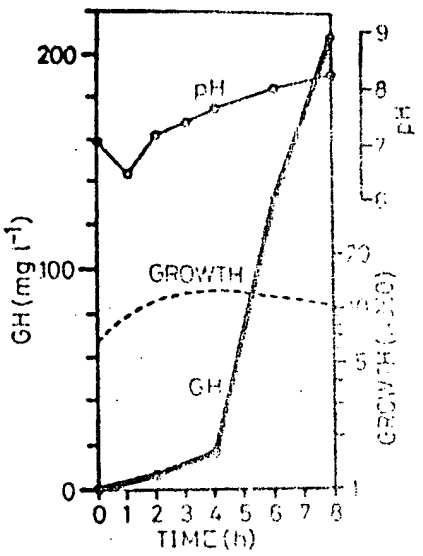

Fig. 8. Secretion of hGH by medium exchange method. The strain MT430 (phGH427) was cultured at 30 °C in 150 ml of medium. After 18 h, cells were harvested and transferred into the same volume of fresh medium. The cultivation was then continued under the same conditions as first culture.

contrast, the accumulation of the hGH in the first culture was 46 mg l^{-1}. The majority of secreted hGH in the second culture was 22 kDa, similar to the result shown in Fig. 7B.

Discussion

In this work, we constructed a host strain for secretion of the heterologous protein, which lacked extracellular alkaline and neutral proteases and possessed *sacQ(BamF)* in the genomic DNA. Using this host, we demonstrated highly efficient secretion of hGH of which gene is located in the secretion vector based on *B. amyloliquefaciens npr.*

We concluded that the strain MT430 constructed here bore *sacQ(BamF)* in the genomic DNA because of the increase of levansucrase activity, morphological change, and loss of the plasmid as well as the transforming ability of the genomic DNA to produce the large halo-forming strain. Since MT400 is a wild-type strain on *rec*, genetic recombination based on DNA-sequence homology might cause the introduction of *sacQ(BamF)*. The location of the gene in the genomic DNA was considered to be *sacQ* due to their homology.

The gene *sacQ* encodes a small polypeptide of 46 amino acid residues which is involved in the transcriptional control of *sacB* (Shimotsu and Henner, 1986). It has also been reported that the expression level of the *β*-galactosidase gene under the control of *sacB* is elevated in the $sacQ^h$ strain (Shimotsu and Henner, 1986). The gene $sacQ^h$ also elevates the extracellular protease levels (Kunst et al., 1974). In this case, however, the mechanism of the enhancement is still unclear. If $sacQ^h$ product

is involved in the transcriptional control of protease genes, the expression of heterologous gene under the control of the protease promoter would be enhanced.

Here, we revealled that the expression of *cat-86* by the *npr* promoter was stimulated 1.7-fold in MT430 compared to the case of MT400. This showed that *sacQ(BamF)* present in the genomic DNA could enhance the expression of the heterologous gene directed by the *npr* promoter, which was located in a multi-copy plasmid, although the stimulation level was not so high. This elevation of *cat-86* expression level might be derived from the increase of the transcripts. The low level of the stimulation (1.7-fold) is considered to be due to the gene dosage of *cat-86* and the strong promoter, by which the transcription level of *cat-86* might be increased sufficiently.

The accumulation level of hGH in 15-h culture of MT430(phGH427) was 5-fold higher than that of MT400(phGH427). As this stimulation level was higher than the case of *cat-86,* it was suggested that something involved in the post-transcriptional stage was also responsible for the increased stimulation. The reduced proteolytic activity in MT430 was considered to contribute mainly to the increased hGH production.

Although the cause of decrease of the residual proteolytic activity by the introduction of *sacQ(BamF)* was unclear, we speculate that the increased production of inactive extracellular alkaline and neutral proteases of MT430 might be involved in the reduction. Power and Adams (1986) have reported that the inactivated subtilisin derived from mutagenized *apr* located in a plasmid, accumulates in membrane as an unprocessed precursor in a *apr* mutant of *B. subtilis* due to the loss of autocatalytic processing of the propeptide. In MT430, such accumulation of the inactive protease-precursors in membrane, which was stimulated by *sacQ(BamF)*, might obstruct the synthesis or secretion of the residual proteases. The precursor hGH encoded by phGH427 also possessed a head portion of the propeptide composed of 21 amino acid residues. We confirmed that this propeptide segment was cleaved by a membrane-associated protease during secretion (Honjo, unpublished data).

Using a 7-l reactor, 80 mg l^{-1} of hGH was secreted into the medium (Fig.6). This result also showed that the production of hGH was in accordance with pH increase and the change of dissolved oxygen (DO) level. Therefore, the phase of hGH secretion could be monitored by pH and DO changes. Using medium exchange method, we achieved the high level accumulation of hGH (210 mg l^{-1}). This suggests that the high concentration cell, fresh medium and restricted aeration permitted the increased secretion and that high density culture might improve the hGH productivity.

As shown in Fig. 6, the accumulation of hGH continued up to 25 h. This continuity was responsible for the increased hGH production since the accumulation level in MT400(phGH427) was reduced after 8–10 h (data not shown). In an 8-h culture, the hGH level of MT400 harboring phGH427 was approximately the same as that of MT430(phGH427).

Previously, we have reported that the secreted 22 K hGH from a low extracellular-protease strain of *B. subtilis* bearing phGH928 contains additional amino acid

sequences derived from junction between the prepropeptide region and hGH gene (50%, Ile-Asn-Ser-Met-hGH; 25%, Asn-Ser-Met-hGH; 25%, Met-hGH) (Nakayama et al., 1987). Since the structure of phGH427 relevant to the expression and secretion of hGH was the same as that of phGH928, the secreted hGH derived from phGH427 might also possess the additional amino acid sequence in the N-terminus.

The authentic natural hGH production using modified this system in high density culture is now in progress.

Acknowledgements

We wish to thank H. Saito, T. Beppu, and Y. Fujita for invaluable suggestions and encouragement. We are grateful to K. Nakajima for supplying hGH cDNA, pituitary derived hGH and antiserum. This work was carried out under the national project 'Research and Development Project of Basic Technologies for Future Industries' supported by the Ministry of International Trade and Industry, Japan.

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Applied and Microbiology Biotechnology

Industrial applications of a cloned neutral protease gene in *Bacillus subtilis*

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Summary. Genes for the α-amylase and neutral protease were cloned from an industrial *Bacillus* isolate, Bsl, onto two separate plasmids and introduced into a *B. subtilis* strain. Both plasmids were stably maintained in this strain. Analysis of the extracellular proteins showed that the plasmid-carrying strain produced predominantly the Bsl α-amylase and neutral protease with few contaminating *B. subtilis* exoenzymes. The presence of high levels of protease enabled the strain to produce considerably more α-amylase when grown on a complex industrial medium rich in protein.

Introduction

The genus *Bacillus* produces a large number of extracellular enzymes, many of which have considerable industrial importance (Priest 1977). A number of these have been cloned to achieve increased enzyme production and more detailed knowledge of secretion and control of gene expression (e.g. Bernier et al. 1983; Cornelis et al. 1982; Fujii et al. 1983; Gray and Chang 1981; Palva 1982; Sashihara et al. 1984).

The first report of cloning in *B. subtilis* of the structural gene for a neutral protease was from *Bacillus stearothermophilus* (Fujii et al. 1983). The cloned fragment was reduced to a 4.5 Md fragment containing the protease gene and was found to be expressed in both *B. subtilis* and *B. stearothermophilus* host strains. Mongkolsuk et al. (1983) reported the cloning of an unspecified protease, when they found that one of two cloned fragments (2.7 kb) from *B. subtilis*, which showed promoter activity during postexponential growth only, also conferred on recipient cells extracellular protease activity. The presence of this recombinant plasmid was found to reduce greatly the ability of the host cells to form spores.

More recently Vasantha et al. (1984) reported the cloning in *B. subtilis* of the genes for an alkaline and neutral protease from *B. amyloliquefaciens* while Yang et al. (1984) have cloned the neutral protease gene of *B. subtilis*. The latter group used deletion derivatives of the cloned gene to replace the wild-type chromosomal copy of the gene and found that this deletion, in combination with a deletion of the alkaline protease gene, completely inhibited protease production but left sporulation unaffected. This strongly suggested that the proteases of *B. subtilis* do not play a role in sporulation. The authors suggested that the only role of proteases was that of scavengers. They commented that the presence of extracellular proteases may limit the accumulation of heterologous gene products due to degradation (Ohmura et al. 1984; Palva et al. 1983).

We decided to exploit the scavenging properties of proteases in order to produce an extracellular protein with a high initial purity. If a foreign bacterial protease gene were introduced into *B. subtilis* its product might degrade the normally excreted proteins of *B. subtilis*. However, if we were to introduce simultaneously into this strain a cloned gene for a different extracellular protein derived from the same foreign bacterium, then this gene product might be resistant to the protease. Hence one should obtain a *B. subtilis* strain which produced very few extracellular proteins other than the protease and the product of the newly cloned gene.

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We decided to test this hypothesis on a *Bacillus* α-amylase gene cloned previously (Corfield et al. 1984). As the relevant fermentation is carried out at a neutral pH we used a neutral protease cloned from the same donor organism.

Materials and methods

Bacterial strains and culture conditions. The bacterial strains used in this study are described in Table 1. Plasmid pPL603b was constructed by P. S. Lovett (personal communication) by inserting a synthetic linker containing a *Bam*H1 site into the *Eco*R1 site of pPL603 (Williams et al. 1981). In order to eliminate the postexponential chloramphenicol acetyl transferase (*cat*) promoter activity of this plasmid (Mongkolsuk et al. 1983), we isolated a spontaneously occurring *B. subtilis* strain 1A46 (pPL603b.1) which was sensitive to chloramphenicol (Cm) at all stages of growth. Bacteria were grown in Luria broth (LB: tryptone, 10 g/l; yeast extract, 5 g/l; sodium chloride, 10 g/l) on an orbital shaker at 37° C. For plasmid-containing strains, the growth medium was supplemented with 10 µg/ml neomycin (Nm), 15 µg/ml tetracycline (Tc), and/or 10 µg Cm/ml, and DM3 regeneration medium (Chang and Cohen 1979) with 25 µg Cm/ml. The industrial medium contained milled barley (4.8%) and malt sprouts (3.2%), adjusted to pH 7.0 with 2 M NaOH.

DNA isolation and purification. Chromosomal DNA was isolated by the method of Lovett and Keggins (1979) with the following modifications. DNA was prepared from a 20 ml overnight culture and the pelleted cells resuspended in 10 ml TES (30 mM Tris pH 8.0, 5 mM EDTA and 50 mM NaCl) pH 7.5. Proteinase K (Sigma) was added to a final concentration of 100 µg/ml and three ether extractions followed the phenol treatment. The DNA precipitated by ethanol was spooled onto a glass rod, air dried and resuspended in 2–3 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA) before dialysis. Plasmids were prepared by the sodium dodecyl sulfate (SDS)-NaCl method of Gryczan and collaborators (1980). For rapid screening the plasmid DNA was isolated according to the single colony lysis method (Marrero and Lovett 1980).

Table 1. *Bacillus* strains

Strain designation	Relevant characteristics	Source
Bsl	*pro*(H)[a]	Industrial isolate
B. subtilis		
1A42	*trp*C2 *thr*5	BGSC[b]
1A46	*trp*C2 *thr*5 *rec*E4	BGSC
1A46(pPL603b.1)	carries Nm^rCm^s plasmid	P. Lovett
SB19	prototroph	—
1A90	*trp*C2 *his*A1 *sac*A321	BGSC
1A274	*npr*	BGSC
1A297	*trp*C2 *asp*T1 *amy*	BGSC
1A297(pVC102)	Bsl α-amylase gene cloned onto pPL603b.1	Corfield et al., 1984
168(pBC16)	Tc^R *ori B. cereus*	Bernhard et al., 1978
B. amyloliquefaciens		
10A3	hsr H(+)	BGSC

[a] *pro*(H): high producer of protease
[b] *Bacillus* Genetic Stock Center, Ohio State University, Dept. of Microbiology, 484 West 12th Avenue, Columbus, Ohio 43210, USA

Restriction, ligation and transformation. Restriction and ligation of DNA was performed using enzymes from Boehringer Mannheim, FRG, according to the manufacturers' instructions. The polyethylene glycol (PEG) induced protoplast transformation procedure of Chang and Cohen (1979) was followed with modifications as described by Corfield et al. (1984). DM3 regeneration medium was modified to include casein as well as Cm in order to facilitate the screening of recombinant strains bearing a protease gene.

Assay of protease activity. The bacteria were grown in LB at 37° C on an orbital shaker. Samples were taken at intervals and centrifuged at 8000 × g for 10 min. The supernatant was assayed for extracellular protease by the azocasein assay of Millet (1970). One milliliter of 0.5% (wt/vol) solution of azocasein in 0.1 M Tris (pH 7.2) was incubated with 0.5 ml of the test culture for 30 min at 37° C. The reaction was stopped by adding 2 ml of a 10% trichloracetic acid (TCA) solution. After standing for 30 min at 4° C, the mixture was filtered through Whatman No. 1 paper, and 1.5 ml of 0.5 N NaOH was added to each 1.5 ml of filtrate. The absorbance was determined at 440 nm. One unit of protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.01 at 440 nm in 30 min at 37° C. Cell concentration was measured as dry weight.

Assay of α-amylase activity. α-Amylase activity was measured by the decrease in iodine staining power of starch digests. Cells were removed by centrifugation and 0.2 ml of appropriately diluted supernatant assayed using 2 ml of 1% hydrolysed Lintner's starch in 0.1 M Na acetate buffer pH 5.5. The reaction was stopped by adding 0.2 ml of digest to 2 ml 0.1 M HCl, after which 0.2 ml of the reaction mixture was added to 2 ml I_2 solution (0.5% I_2, 0.5% KI). Reagent blanks were run simultaneously and absorbance read at 660 nm against water. One unit of α-amylase activity was defined as that which resulted in a 1% reduction in the absorbance of the I_2-starch complex at 660 nm in one minute.

Enzyme inhibition. The protease preparation was treated with 5 mM EDTA at 37° C for 30 min or with 1 mM phenylmethylsulfonylfluoride (PMSF) at 0° C for 1 h. Samples were taken to assess the azocasein hydrolytic activity at 37° C by the procedure referred to earlier.

Optimum pH. Sodium citrate buffers (50 mM; ranging from pH 5 to 6) and Tris-HCl buffers (50 mM; ranging from pH 7 to 10) were used to determine an optimal pH value for protease activity. Culture supernatants, which had been dialysed against each buffer with the addition of 5 mM $CaCl_2$, were mixed with an azocasein solution (1% dissolved in the same buffer) and protease activity measured.

Gel electrophoresis. Agarose gel electrophoresis of DNA samples was performed on a horizontal apparatus as described by Canosi et al. (1982). SDS-polyacrylamide gel electrophoresis of protein preparations was performed according to Laemmli (1970). The method of Weber and Osborn (1969) was used to

determine the molecular weight of the protein. Gelatin zymograms to determine the activity of the proteases were performed according to Heussen and Dowdle (1980). Gels were scanned using an LKB 2202 Ultroscan laser densitometer.

DNA hybridization. Chromosomal DNA of the industrial isolate Bsl, *B. subtilis* strains 1A46 and SB19, and *B. amyloliquefaciens* was digested with *Bgl*II or *Hind*III, electrophoresed in agarose and transferred to nitrocellulose paper (Schleicher and Schuell) by the method of Southern (1975). The DNA was hybridized to a ^{32}P-labelled pSR51 probe, prepared by nick translation (Rigby et al. 1977). The nitrocellulose was washed and exposed to X-ray film for detection of homologous sequences.

Plasmid stability. Plasmid-carrying bacterial strains were plated on LB agar containing casein, Cm and Nm. Single colonies showing casein hydrolysis were resuspended in LB, stored at 4°C, and appropriate dilutions plated on the above medium. Colonies exhibiting no segregation of Cm, Nm or *npr*$^+$ were inoculated into 25 ml of LB with or without Cm and Nm at a concentration of 1.2×10^4 cfu/ml and shaken at 37°C. After each 15 generations of growth, the cultures were diluted as above. The procedure was continued for 60 generations. At appropriate time points, aliquots of the cultures were enumerated on casein-containing LB agar with or without antibiotics.

Results

Cloning of a protease gene in B. subtilis

Protease assays were performed on a variety of *B. subtilis* strains to obtain a suitable recipient for the detection of a cloned protease gene. Three strains, 1A46, 1A274 and 1A90, were identified which had reduced levels of extracellular proteases and could be transformed successfully by the protoplast method. The strain exhibiting the highest transformation efficiency, 1A46, was chosen as the recipient in cloning experiments. In order to clone fragments of Bsl DNA into *Bam* HI-linearized pPL603b.1, donor DNA was digested with *Bgl* II, as it proved to be resistant to *Bam* H1, ligated to the plasmid and transformed into *B. subtilis* 1A46. Transformants carrying promoter-bearing recombinant plasmids were selected on DM3 regeneration medium containing Cm and casein. Among 1 271 colonies isolated (frequency of 6×10^3 recombinants/µg DNA), one colony showed a zone of casein hydrolytic activity, was resistant to Cm and Nm, and contained a plasmid, designated pSR51. Transformation of 1A46 with this plasmid yielded Cm^r Nm^r *npr*$^+$ colonies at an efficiency of 4.2×10^4/µg DNA, indicating that the ability to produce protease was associated with the recombinant plasmid. Restriction endonuclease analysis of pSR51 showed it to have an insert of 4.6 kb giving a total size of 10 kb (Fig. 1).

To prove conclusively that the insert in pSR51 was derived from Bsl DNA, Southern gel analysis was performed using ^{32}P-labelled pSR51 as a probe to hybridize to *Bgl* II and *Hind* III digested

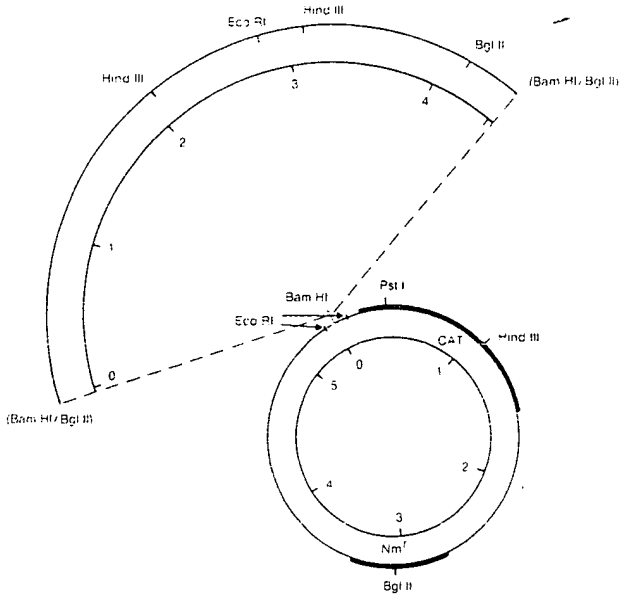

Fig. 1. Restriction endonuclease map of pSR51. Plasmid pPL603b was restricted with *Bam* H1 and ligated to *Bgl* II fragments from *Bacillus* Bsl. The Arabic numerals inside the circles indicate the molecular size in kb

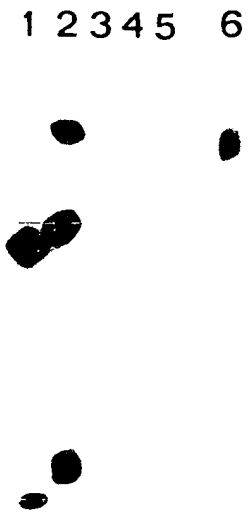

Fig. 2. Hybridization of nick-translated pSR51 to *Bgl* II- and *Hind* III-digested chromosomal DNA. Lane 1, *Bgl* II-digested Bsl DNA; 2, *Hind* III-digested Bsl DNA; 3, 4 and 5, *Bgl* II-digested 1A46, 10A3 and SB19, respectively; 6, pSR51

Bsl DNA. The probe hybridized to *Bgl* II fragments of 3.8 and 0.8 kb, as expected from the map of pSR51, and to *Hind* III fragments of 7.8, 4.5 and 1.1 kb, the last of which represented the internal protease gene fragment (Fig. 2). No hybridization occurred to *B. subtilis* strains or *B. amyloliquefaciens* 10A3.

Stability of hybrid plasmid, pSR51

As many hybrid plasmids in *B. subtilis* exhibit considerable structural and segregational instability (Lopez et al. 1984; Uhlén et al. 1981), the stability of pSR51 was investigated. To determine whether the host cell recombination system played a role in stability, pSR51 was transformed into an otherwise isogenic pair of *rec*$^+$ (1A42) and *rec*$^-$ (1A46) strains. In the presence of antibiotics no loss of protease activity occurred in either strain. In the absence of antibiotics pSR51 showed only 4% segregation over 60 generations in the *rec*$^-$ strain 1A46, whereas 99% of the *rec*$^+$ 1A42 cells lost the plasmid within 30 generations. Thus this hybrid plasmid showed no structural instability and did not segregate in a *rec*$^-$ strain.

Production of the Bsl protease in B. subtilis

The level of protease production in LB by 1A46 (pSR51) was compared with the donor Bsl and recipient 1A46 using the azocasein protease assay. Over a period of 24 h the cloned gene directed the production of 156 U of neutral protease/mg dry cell weight compared with 79 U of total proteases/mg dry cell weight produced by Bsl. The recipient 1A46 produced only 11 U/mg dry cell wt. As 85% of Bsl proteases consist of neutral protease (see below) this represents a 2.3 fold increase of the cloned gene product over the donor. Sporulation frequency was not greatly affected by the presence of the recombinant plasmid in contrast to the 90% reduction found by Mongkolsuk et al. (1983). However the onset of sporulation was delayed by 4 h which may be a reflection of the slower growth rate of the strain containing the recombinant plasmid.

Characterization of the cloned protease

The azocasein hydrolytic properties of the cloned protease were compared with the total activities of the proteases of Bsl in the presence or absence of PMSF, a serine protease inhibitor, or EDTA, a neutral protease inhibitor. The results showed that after incubation of culture supernatants with the inhibitors for 1 h and 30 min, respectively, the protease activity in 1A46 (pSR51) was completely inhibited by EDTA but only slightly by PMSF (97% activity remaining). The protease activity of Bsl was shown to be due to both neutral and serine proteases as EDTA inhibited 85% and PMSF 11% of the activity.

The pH optima for protease activity of 1A46 (pSR51) and Bsl were compared to confirm that we had cloned the gene for a neutral protease from the mixture of protease genes of Bsl. Maximal activity of the cloned protease from 1A46 (pSR51) and the total proteases of Bsl occurred at pH 7.2. In the presence of EDTA the remaining proteolytic activity of Bsl had an optimum at pH 9 and 10, presumably due to serine protease activity.

In order to demonstrate that the properties of the neutral protease had not been altered as a result of the cloning, extracellular proteins produced by Bsl and pSR51 in 1A46 or 1A42, were characterized by SDS-polyacrylamide gel electrophoresis (Fig. 3). A protein with a molecular weight of 40,500 daltons, absent in 1A46 and

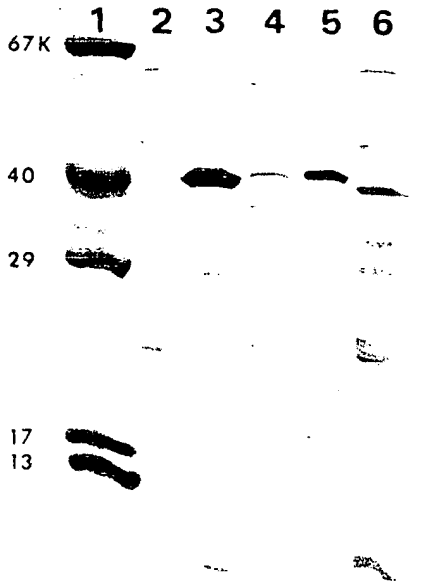

Fig. 3. Polyacrylamide gel electrophoresis of culture supernatants. Strains were grown for 20 h in LB and aliquots of the supernatants were run on an SDS-polyacrylamide gel pH 8.6 at 20 mA for 4 h. The gel was stained with 0.05% Coomassie Brilliant Blue R250. Lane 1, molecular weight markers; 2, 1A46; 3, 1A46(pSR51); 4, Bsl; 5, 1A42(pSR51); 6, 1A42

1A42 was present in high concentration in 1A46 (pSR51) and 1A42 (pSR51) and comigrated with a protein from the donor Bsl, which exhibited protease activity when electrophoresed in a polyacrylamide gel containing 0.1% gelatin and subsequently allowed to digest at 37° C in a 0.1 M glycine-NaOH buffer pH 7.2 (results not shown).

It was also clear from these results that the cloned Bsl protease was capable of degrading the extracellular proteins of *B. subtilis* strains 1A46 and 1A42. To determine whether an extracellular protein cloned from the same donor organism would be resistant to this protease, we incubated cell supernatants of 1A46 (pSR51) with cell supernatants of *B. subtilis* strains harbouring a hybrid plasmid, pVC102. This plasmid was constructed by ligating a Bsl *Bgl* II fragment carrying the α-amylase gene with *Bam* H1 linearized pPL603b.1 (Corfield et al. 1984). The α-amylase was shown to be protease resistant even after 90 min incubation at 37° C.

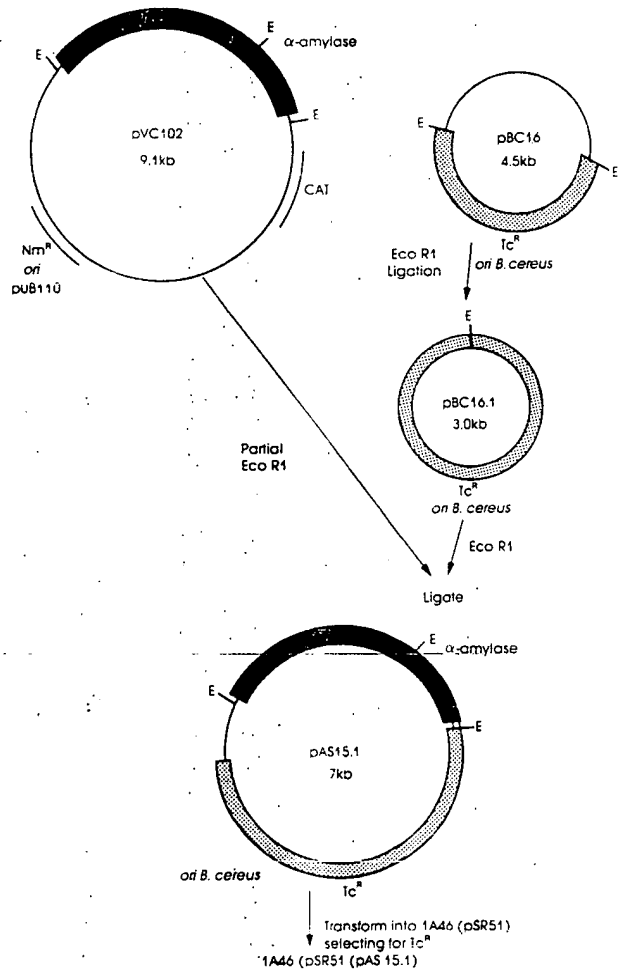

Fig. 4. Construction of plasmid pAS15.1. Partial *Eco*R1 digests of pVC102 were ligated to *Eco*R1 digests of pBC16.1 to give an unstable plasmid resistant to Cm and Tc. This intermediate plasmid underwent a spontaneous deletion of 4.9 kb to give pAS15.1 which is resistant to Tc and encodes the α-amylase gene of pVC102

Construction of a B. subtilis strain containing both the Bsl protease and α-amylase genes

We decided not to subclone the Bsl protease gene onto pVC102, the hybrid plasmid carrying the Bsl α-amylase gene, as this might well result in undesirable deletions. We therefore introduced the genes into *B. subtilis* on two separate plasmids. Our strategy is outlined in Fig. 4. The cloning of the Tc^R gene from pBC16.1 onto pVC102 resulted in the deletion of 4.9 kb of pPL603b.1 DNA. The resulting plasmid, pAS15.1, therefore expressed Tc^R but not Cm^R or N^R and replicated from the introduced *B. cereus* origin. When pAS15.1 was transformed into the *B. subtilis* strain carrying the cloned protease gene, 1A46 (pSR51), the two plasmids were stably maintained in the presence of Cm and Tc. Analysis of the extracellular proteins by SDS-polyacrylamide gel electrophoresis showed that 1A46(pSR51)(pAS15.1) produced predominantly the protease and α-amylase enzymes, whereas 1A46 with or without the original vector pPL603b.1 produced many high molecular weight extracellular proteins (Figs. 5 and 6A).

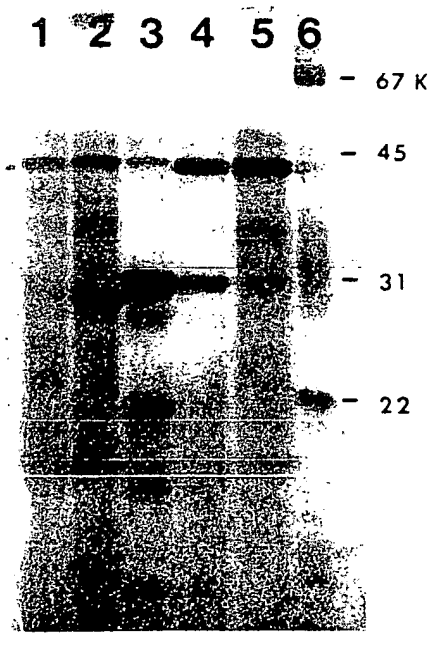

Fig. 5. Polyacrylamide gel electrophoresis of supernatants from cultures grown for 48 h in LB. Lane 1, 1A46; 2, 1A46(pPL603b); 3, 1A46(pSR51); 4, 1A46(pSR51)(pAS15.1); 5, 1A46(pAS15.1); 6, molecular weight markers

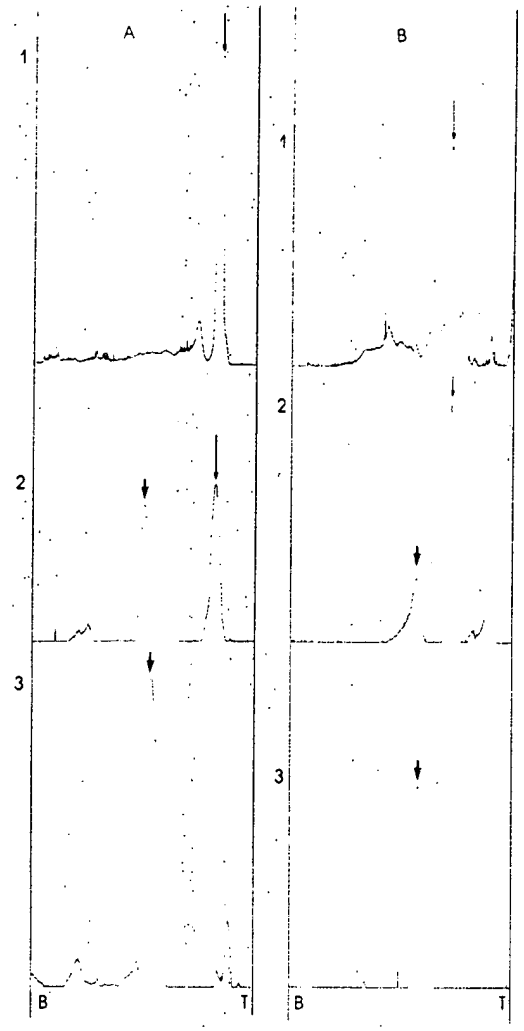

Fig. 6. Scans of polyacrylamide gels of supernatants from cultures grown for 48 h in (**A**) LB and (**B**) industrial medium. *Lane 1.* 1A46(pAS15.1); 2. 1A46(pSR51) (pAS15.1); 3. 1A46(pSR51). • denotes protease peak; – denotes amylase peak

Mixed fermentations of the two strains each containing the single plasmid also showed degradation of most of the extracellular proteins other than the α-amylase and protease (results not shown).

Production of protease and amylase in industrial media

The exploitation of *B. subtilis* for the export of foreign proteins is often hampered by the fact that the protease deficient hosts used to allow efficient production of heterologous gene products do not grow well on industrial substrates rich in proteins (Nybergh 1984). The present system was considered to be a candidate for overcoming this problem and the enzyme yields in a complex industrial medium were therefore investigated. The protease levels increased 5-fold when 1A46(pSR51) was grown in such a medium (Fig. 7), and it was of interest to note that activity increased up to 72 h, whereas in LB maximal activity was achieved after 18–24 h.

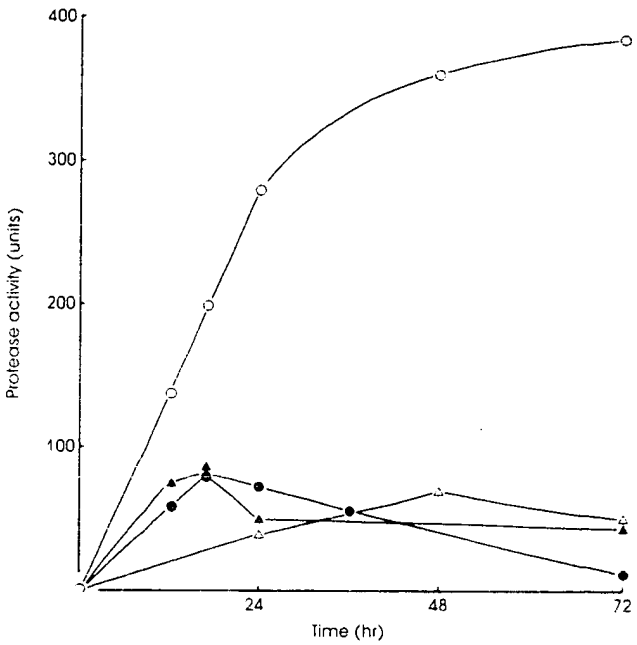

Fig. 7. Production of extracellular protease by the industrial donor strain, Bsl and 1A46(pSR51) in LB *(closed symbols)* and industrial medium *(open symbols)*: △ Bsl; ○ pSR51

Table 2. α-Amylase and protease production in complex industrial medium and LB + 2% starch[a]

Plasmid[b]		Industrial medium		LB + 2% starch	
	Time h	Protease U	α-Amylase U	Protease U	α-Amylase U
pAS15.1	24	24	33	5	17
	48	30	143	5	39
	72	32	253	6	64
(pSR51)(pAS15.1)	24	168	6	25	22
	48	222	121	25	21
	72	258	408	23	52
pSR51 + pAS15.1 (Mixed fermentation)	24	145	37	65	15
	48	189	62	73	17
	72	181	374	71	41
pSR51	24	192	4	75	1
	48	360	10	61	2
	72	396	12	23	4

[a] Results represent the average of 3 to 5 experiments
[b] All plasmids were in 1A46

A comparison was made of the levels of α-amylase and protease when strains of 1A46 carrying various combinations of pAS15.1 and pSR51 were grown on a complex industrial medium or on LB + 2% starch (Table 2). In all cases enzyme levels were from 3- to 17-fold higher when strains were grown in the industrial medium, despite the fact that growth characteristics were essentially unchanged. Although the copy number of pAS15.1 decreased when pSR51 was present (results not shown), α-amylase levels in 1A46(pSR51)(pAS15.1) when grown in industrial medium were approximately 1.6-fold higher after 72 h than 1A46(pAS15.1). This increase was not seen in the LB medium. A similar increase of 1.5-fold was achieved in mixed fermentations. In the industrial medium protease degradation of high molecular weight extracellular proteins, other than Bsl α-amylase, was evident (Fig. 6B).

Discussion

This paper confirms the hypothesis that a *B. subtilis* strain carrying a foreign protease gene as well as another extracellular protein gene, cloned from the same donor strain, would produce predominantly these two extracellular proteins. This would be expected to occur if the protease degraded the *B. subtilis* exoproteins but not the product of the second cloned gene. Using the promoter-selection vector pPL603b.1 we cloned a neutral protease from an industrial strain of *Bacillus*, Bsl, and verified that its pH optimum and molecular weight had not been altered during the cloning process. This was important as any alteration might change its proteolytic specificity.

Southern gel analysis proved that the gene was indeed derived from Bsl DNA, but lack of hybridization to *B. subtilis* and *B. amyloliquefaciens* indicated that the Bsl protease gene has little homology with proteases of these species.

A stable, high copy number hybrid plasmid carrying the protease gene was required. Despite its size (10 kb), plasmid pSR51 was found to be stable over 45 generations in a *rec*E strain, 1A46, showing a mere 4% segregation in the absence of selective antibiotics. However, when it was transferred to 1A42, an isogenic strain which is rec^+, 99% plasmid loss occurred within 30 generations. This is in contrast to Uhlén et al. (1981), who found that in the absence of selective pressure, loss of plasmid pC194 carrying fragments of the *B. subtilis* bacteriophage Ø105 occurred to the same extent in rec^+ and rec^- hosts. We have determined that the vector plasmid, pPL603b, does not segregate at all in either 1A46 or 1A42 over 45 generations (results not shown). Unlike many recombinant plasmids in *B. subtilis* (Lopez et al. 1984; Uhlén et al. 1981; our unpublished data), pSR51 showed no structural instability either in the presence or absence of selective antibiotics.

The enzyme we chose to be co-produced with the protease was the α-amylase of Bsl, as we had previously cloned its structural gene. The enzymatic activity of the cloned Bsl α-amylase proved to be resistant to the cloned Bsl neutral protease. This was found either when supernatants of 1A46(pSR51) and 1A46(pAS15.1) were incubated together or when the two strains were grown in a mixed culture. We then constructed a strain containing both these genes on two separate plasmids, pAS15.1 and pSR51. Analysis of the extracellular proteins by SDS-polyacrylamide gel electrophoresis showed that 1A46(pSR51)(pAS15.1) grown on LB + 2% starch produced predominantly the protease and α-amylase enzymes with few contaminating *B. subtilis* exoproteins. The same effect was achieved in mixed fermentations of 1A46(pSR51) and 1A46(pAS15.1).

As it has been shown that a protease-deficient *B. subtilis* carrying a hybrid α-amylase plasmid produces less enzyme when grown on a complex industrial medium than on a synthetic medium (Nybergh 1984), an investigation of the behaviour of the various plasmid-carrying strains in a complex industrial medium was undertaken. In all cases α-amylase levels were 3- to 9-fold higher than in LB + 2% starch. It was of interest to note that 1A46(pSR51)(pAS15.1) produced 1.6-fold more α-amylase than 1A46(pAS15.1) despite the lower α-amylase plasmid copy number in the former. The increased levels of α-amylase when the strain was grown in a complex industrial medium in the presence of high levels of protease may be due to the scavenger properties of the protease. This effect could also be achieved in a mixed fermentation but the levels of α-amylase were higher when both the α-amylase and protease plasmids were present in one strain. It is anticipated that once a stable, high-copy-number hybrid plasmid producing both the Bsl neutral protease and α-amylase has been isolated, that enzyme yields on industrial media will improve further.

Protease "clean-up" of *B. subtilis* extracellular proteins was also observed in the industrial medium. This system therefore facilitates the efficient production of an extracellular enzyme with a high initial purity.

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Gene Encoding a Minor Extracellular Protease in *Bacillus subtilis*

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The gene for a minor, extracellular protease has been identified in *Bacillus subtilis*. The gene (*epr*) encoded a primary product of 645 amino acids that was partially homologous to both subtilisin (Apr) and the major internal serine protease (ISP-1) of *B. subtilis*. Deletion analysis indicated that the C-terminal 240 amino acids of Epr were not necessary for activity. This C-terminal region exhibited several unusual features, including a high abundance of lysine residues and the presence of a partially homologous sequence of 44 amino acids that was directly repeated five times. The *epr* gene mapped near *sacA* and was not required for growth or sporulation.

The gram-positive, sporeforming bacterium *Bacillus subtilis* produces and secretes proteases, esterases, and other kinds of exoenzymes at the end of the exponential phase of growth (15). The principal extracellular proteolytic enzymes, the alkaline (subtilisin) and neutral (metallo-) proteases, are encoded by the *apr* and *npr* genes, respectively (19, 21, 23). Double mutants bearing null mutations in both genes are substantially blocked in extracellular protease production but still secrete 2 to 4% of the wild-type level of protease (10; our unpublished results). We have been interested in identifying additional protease genes responsible for the residual extracellular protease production by Apr^-, Npr^- double mutants. Here we report the cloning of a new protease gene whose product, a protease sensitive to phenylmethylsulfonyl fluoride (PMSF) and EDTA, shows significant homology to subtilisin.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* GP208 (*ΔaprA ΔnprE ΔispA metC amyE*) is a derivative of the *B. subtilis* 168 strain IS75. Plasmids pBD64 or pUC19 and pBR322 were used for cloning in *B. subtilis* and *Escherichia coli*, respectively. The *cat* gene was obtained from plasmid pM1101 (24). *B. subtilis* strains were grown on tryptose blood agar base (Difco Laboratories) or minimal glucose medium and were made competent by the procedure of Anagnostopoulos and Spizizen (1). *E. coli* JM107 was grown and made competent by the procedure of Hanahan (7). Plasmid DNA from *B. subtilis* and *E. coli* was prepared by the alkaline lysis method of Birnboim and Doly (2). Plasmid DNA transformation in *B. subtilis* was performed as described by Gryczan et al. (6).

Enzymes. Restriction enzymes, T4 DNA ligase, *Bal*31 exonuclease, and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim.

DNA isolation and gene library characterization. Isolation of *B. subtilis* chromosomal DNA was done as described previously (4). Total DNA from GP208 (100 μg) was digested with *Sau*3A (4 U) at 37°C for 10 min. The partially digested DNA was run on an 0.8% agarose gel. Fragments of 3 to 7 kilobases (kb) were electroeluted and ligated to *Bgl*II-digested pEc224 that had been treated with alkaline phosphatase (1 U) for 30 min at 37°C. The ligation was done at a ratio of insert to vector DNA of 4:1. The ligation mix was incubated at 16°C overnight and transformed into JM107.

Restriction fragments to be sequenced were ligated into appropriate sites of M13 vectors. DNA sequencing was performed by the dideoxy chain termination method (17).

Mapping of *epr* gene. Mapping of the *epr* locus was performed by PBS1 transduction (9) with a lysate from *B. subtilis* GP208(pNP7 integrated) Cm^r. Cm^r transductants were scored for linkage to several loci from the Dedonder set of reference strains (3).

Protease assays. Protease assays were performed by adding 100 μl of culture supernatant to 900 μl of 50 mM Tris (pH 8)–5 mM $CaCl_2$, with 10 mg of Azocoll (Sigma). The solutions were incubated at 37°C for 30 min with constant shaking. The reactions were then centrifuged to remove the insoluble Azocoll, and the A_{520} of the solution was determined. Inhibitors were preincubated with the reaction mix for 5 min at 37°C.

[^{14}C]casein was used to measure low amounts of residual protease. Culture supernatant (100 μl) was added to 100 μl of 50 mM Tris (pH 8)–5 mM $CaCl_2$ containing 10^5 cpm of [^{14}C]casein (New England Nuclear). The solutions were incubated at 37°C for 30 min. The reaction mixes were then placed on ice, and 20 μg of bovine serum albumin was added as carrier protein. Cold 10% trichloroacetic acid (600 μl) was added, and the mix was kept on ice for 10 min. The solutions were centrifuged to spin out the precipitated protein, and the radioactivity in the supernatants was counted in a scintillation counter. Up to 20 to 30% degradation remained in the linear range of the assay.

RESULTS

Cloning an additional protease gene from *B. subtilis*. To identify additional extracellular protease-encoding genes, we used a high-copy-number vector to construct a library of cloned *B. subtilis* DNAs from a triple mutant that contained deletions of the genes for the three known proteases: subtilisin (*apr*), neutral protease (*npr*), and the major intracellular serine protease (*isp*). We reasoned that amplification of the additional protease gene(s) on a high-copy-number vector would increase protease levels sufficiently to produce a halo around colonies containing a cloned protease gene.

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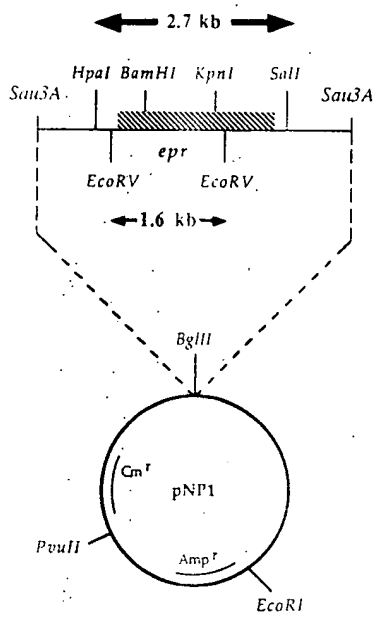

FIG. 1. Restriction map of pNP1 containing the *epr* gene cloned in pEc224.

To construct a gene bank, total chromosomal DNA was isolated from strain GP208 (*Δapr Δnpr Δisp*) and partially digested with *Sau*3A. Size-fractionated fragments (3 to 7 kb) were cloned into the *Bgl*II site of pEc224, a shuttle vector made from pBR322 and pBD64 joined at the *Eco*RI and *Pvu*II sites. Transformation of competent cells of *E. coli* JM107 resulted in more than 1,200 colonies. Plasmid screening showed that 90% of the colonies contained plasmids with inserts of an average size of about 4 kb; however, none of the *E. coli* transformants produced halos on casein plates.

To screen for protease activity in *B. subtilis*, the 1,200 *E. coli* colonies were pooled into groups of 100 (G1 to G12). The groups were grown in liquid culture (LB and ampicillin [50 μg/ml]), and miniprep plasmid DNA prepared from each group was used to transform *B. subtilis* GP208. Approximately 5% of the transformants from one of the pools (G11) produced halos on casein plates.

Cleavage of the plasmid DNA with restriction enzymes showed that all the transformants producing halos had a plasmid with the same-sized DNA insert (about 4 kb). A restriction map of one such plasmid, pNP1, is shown in Fig. 1. The protease secreted by GP208 cells containing pNP1 was sensitive to both PMSF (1 mM) and EDTA (10 mM).

Nucleotide and deduced amino acid sequences of the protease gene. Subcloning and deletion experiments established that all or most of the protease gene was contained on the 1.6-kb *Eco*RV fragment (Fig. 1). Determination of the nucleotide sequence of the 1.6-kb *Eco*RV fragment revealed an open reading frame (ORF) which covered almost the entire fragment, starting 450 base pairs (bp) from the left end and proceeding through the right end (Fig. 2). Comparison of the deduced amino acid sequence with other amino acid sequences in GENBANK indicated that the protein encoded by the ORF had strong homology (approximately 40%) to both subtilisin (19) and ISP-1 (11) from *B. subtilis* 168 (Fig. 3A and B). The most probable initiation codon for this protease gene is the ATG at position 1 in Fig. 2. This ATG (second codon in the ORF) was preceded by an excellent *B. subtilis* ribosome-binding site (AAAGGAGATGA), which had a calculated ΔG of −16.4 kcal (20). In addition, the first 26 amino acids following this Met resembled a typical *B. subtilis* signal sequence, with a short sequence containing two positively charged amino acids, followed by 15 hydrophobic amino acids, a helix-breaking proline, and a typical Ala-X-Ala signal peptidase cleavage site (13). Even though the 1.6-kb *Eco*RV fragment was sufficient for protease activity, the ORF continued past the end of the downstream *Eco*RV site. To locate the 3′ end of the gene, the DNA sequence of the overlapping *Kpn*I-*Sal*I fragment was determined (Fig. 1). As shown in Fig. 2, the end of the ORF was found 717 bp downstream from the *Eco*RV site, and the entire *epr* gene was found to encode a 645-amino-acid protein, of which approximately the first 380 amino acids were homologous to subtilisin (Fig. 3). The final approximately 240 amino acids were apparently not essential for proteolytic activity, since subcloning experiments with the 1.6-kb *Eco*RV fragment indicated that the information encoded in the first 405 amino acids was sufficient for protease activity.

Structure of the Epr protein. In vitro transcription-translation experiments were used to confirm the size of the gene. Addition of *B. subtilis* or *E. coli* plasmid DNA containing the 2.7-kb *Hpa*I-*Sal*I fragment with the entire *epr* gene (pNP3 or pNP2) to an S30-coupled transcription-translation system (New England Nuclear) resulted in the synthesis of a protein of approximately 75,000 daltons (Fig. 4A). (Additional proteins of 60,000 and 34,000 daltons were also seen and presumably represented processed or degraded forms of the 75,000-dalton protein.) This size was in reasonable agreement with the predicted molecular weight for the primary product of 69,702.

The homology of the *epr* protease to subtilisin throughout the amino-terminal half of Epr suggests that Epr might also be produced as a preproenzyme with a prosequence of similar size to that found for subtilisin (70 to 80 amino acids). If true, and if there were no additional processing, this would argue that the mature Epr enzyme has a molecular weight of ca. 58,000. Examination of culture supernatants, however, indicated that the protein had a molecular weight of about 34,000. Comparison by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels of the proteins secreted by *B. subtilis* strain GP208 containing a plasmid with the *epr* gene (pNP3 or pNP5) or just the parent plasmid alone (pBs81/6) showed that the 2.7-kb *Hpa*I-*Sal*I fragment (Fig. 1) cloned in pNP3 directed the production of proteins of about 34,000 and 38,000 daltons (Fig. 4B), whereas the 1.6-kb *Eco*RV fragment cloned in pNP5 in the same orientation (Fig. 1) directed production of just the 34,000-dalton protein (data not shown). The two proteins appeared to be different forms of the Epr protease, resulting from either processing or proteolytic degradation. Clearly, the 1.6-kb *Eco*RV fragment, which lacks the 3′ third of the *epr* gene, is capable of directing the production of an active protease similar in size to that observed when the entire gene is present. This suggests that the protease normally undergoes C-terminal processing.

Location of *epr* on the *B. subtilis* chromosome. To map *epr* on the *B. subtilis* chromosome, we introduced a drug resistance marker into the chromosome at the site of the *epr* gene and used phage PBS1-mediated transduction to determine the location of the insertion. A 1.3-kb *Eco*RI fragment containing a chloramphenicol acetyltransferase (*cat*) gene was cloned into the unique *Eco*RI site on an *E. coli* plasmid containing the *epr* gene (pNP2, Fig. 5). The resulting plasmid (pNP7) was used to transform *B. subtilis* GP208, and chloramphenicol-resistant transformants were selected. Since the

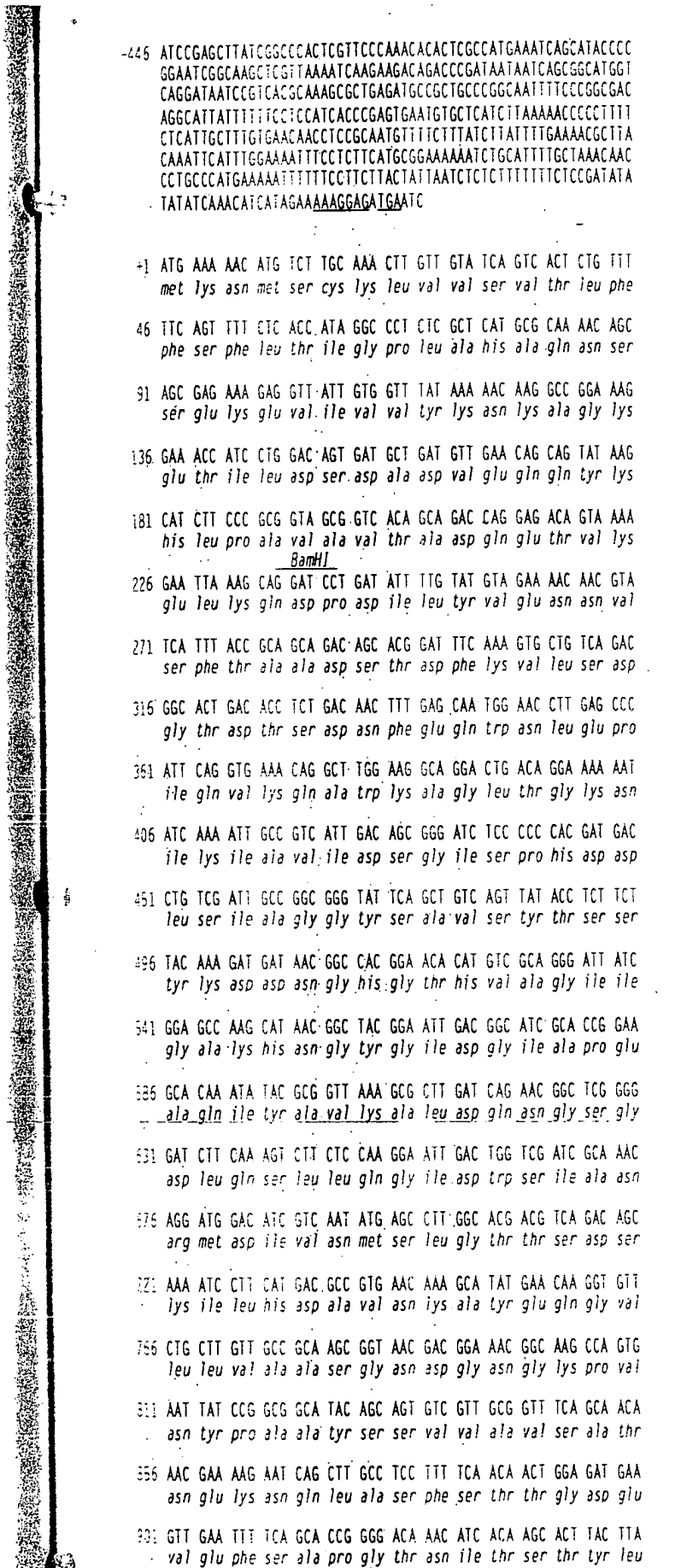

FIG. 2. Nucleotide and deduced amino acid sequences of the *epr* gene. Nucleotides are numbered starting with the A of the presumed initiation codon ATG. The putative ribosome-binding site and a putative transcription terminator are underlined. The partially homologous, directly repeated sequences in the C-terminal domain are indicated in brackets. The translational stop codon (...) is indicated.

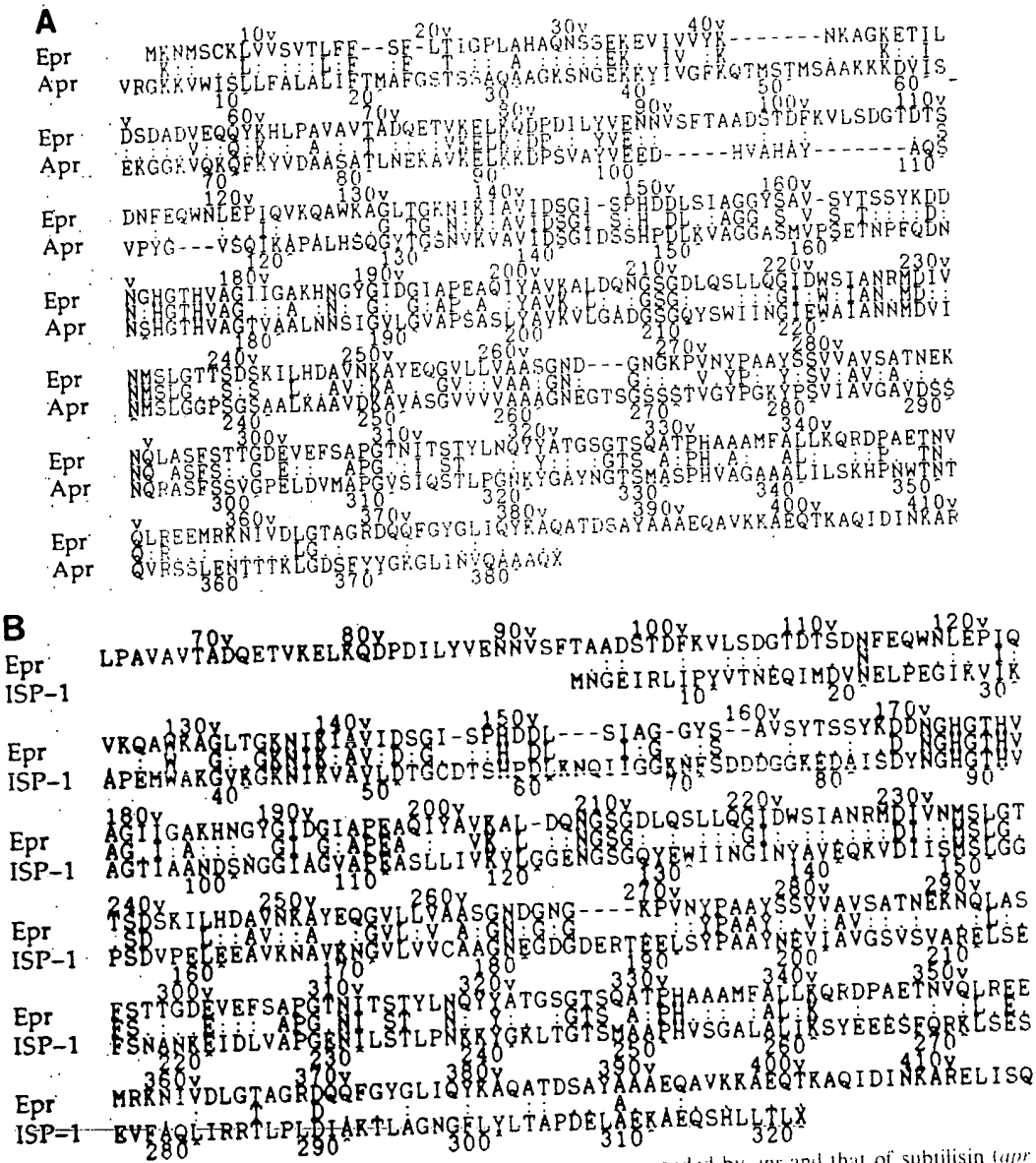

FIG. 3. (A) Comparison of the deduced amino acid sequence of the protease encoded by *epr* and that of subtilisin (*apr* gene product). Identical amino acids are indicated by the appropriate single-letter code; similar amino acids are designated with a colon. Unrelated amino acids are designated with a dot or blank. The final 235 amino acids of Epr are not shown. (B) Comparison of homology between the deduced amino acid sequences of *epr* and ISP-1. The first 61 and final 230 amino acids of Epr are not shown.

plasmid cannot replicate autonomously in *B. subtilis*, the Cm^r transformants were expected to arise by virtue of a single, reciprocal recombination event between the cloned *epr* gene on the plasmid and the chromosomal copy of the gene. Southern hybridization confirmed that the *cat* gene had integrated in the chromosome at the site of the cloned *epr* gene. Mapping experiments indicated that the inserted *cat* gene and *epr* gene were tightly linked to *sacA321* (77% cotransduction), weakly linked to *purA16* (5% cotransduction), and unlinked to *hisA1*. These findings suggest that the *epr* gene is situated near *sacA* in an area of the genetic map not known to contain any other protease genes.

***epr* is a nonessential gene.** To examine the function of the *epr* gene, we constructed a deletion in the 5′ end of the cloned gene and then replaced the wild-type gene in the chromosome with the in vitro-created deletion mutation. A deletion mutation was created in *epr* by cleavage at the unique *Bam*HI site in pNP2 and digestion with *Bal*31 (Fig. 5). After religation and transformation, one plasmid (pNP8) was selected which contained a 2.3-kb insert instead of the original 2.7-kb insert. (As predicted, deletion of the 400 bp of *epr* generated a fragment that, when cloned on a *Bacillus* plasmid, no longer produced an active protease, as measured by the appearance of a halo on casein plates.)

To introduce the deleted gene into the *B. subtilis* chromosome, the *cat* gene described above was introduced into the *Eco*RI site on pNP8 to create pNP9. This *E. coli* plasmid was used to transform *B. subtilis* GP208 to Cm^r, and 70% of the resulting transformants had a very slight halo and 30% had no halos on casein plates. The absence of a halo is the result of a double crossover event between homologous chromosomal DNA and a concatemer of the plasmid DNA (19):

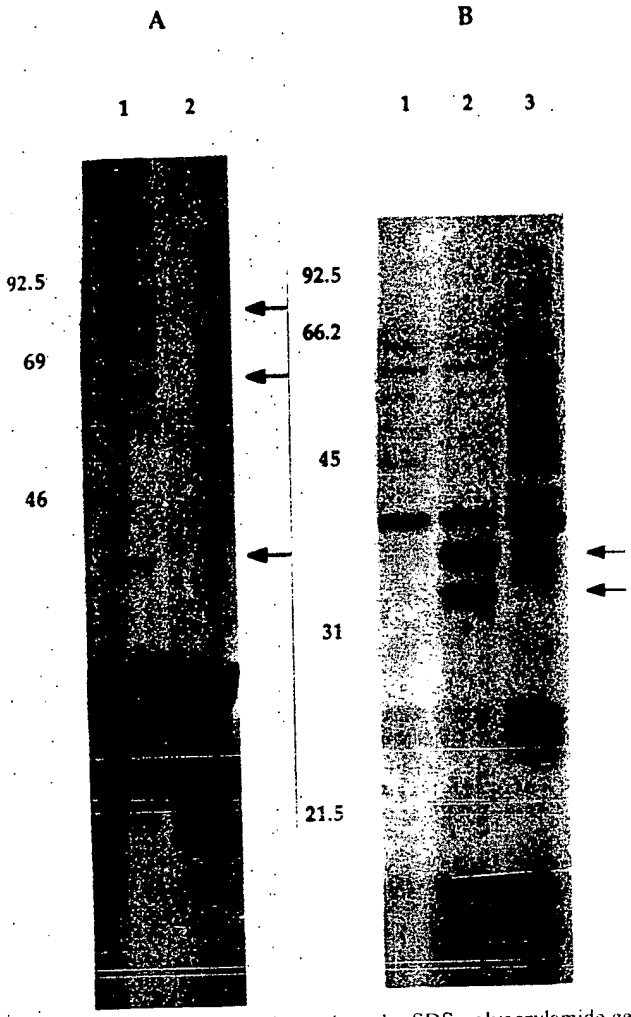

FIG. 4. Analysis of protein products by SDS-polyacrylamide gel electrophoresis. (A) Autoradiograph of [^{35}S]methionine-labeled proteins synthesized in an S30-coupled transcription-translation system and electrophoresed on a 7.5% polyacrylamide–SDS gel. Lanes: 1, pNP2; 2, pUC19. The arrows indicate the 75-kDa, 60-kDa, and 34-kDa proteins synthesized from pNP2. Molecular mass standards are given in kilodaltons. The thickest band in both lanes corresponds to β-lactamase of pUC19. (B) Electrophoresis of culture supernatants. Cultures were grown in LB with chloramphenicol (5 μg/ml). Three hours after the onset of stationary phase, culture supernatants were removed, boiled in SDS sample buffer, electrophoresed on a 7.5% polyacrylamide–SDS gel, and stained with Coomassie brilliant blue. Lanes: 1, pBs81/6; 2, pNP3; 3, pNP6 (pNP1 with the 1.6-kb *Eco*RV fragment deleted). The arrows indicate the 38-kDa and 34-kDa proteins synthesized from pNP3. Molecular mass standards are given in kilodaltons.

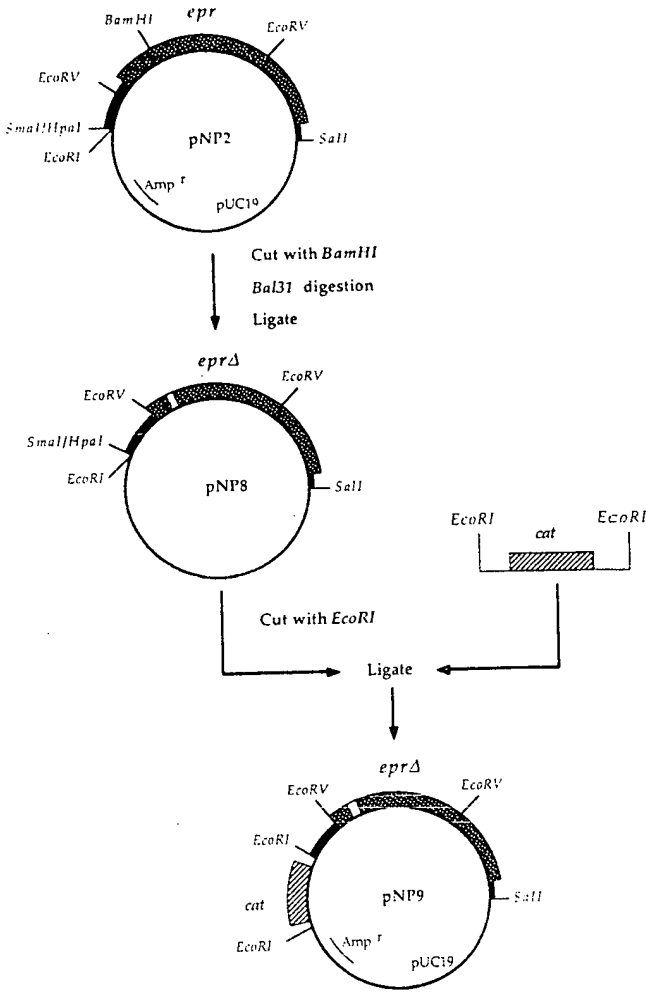

FIG. 5. Construction of plasmid pNP9 containing a deletion mutation of the *epr* gene.

these strains contained the *E. coli* replicon and *cat* gene flanked by two copies of the deleted *epr* gene. To screen for a strain that had undergone a recombination event between the two copies of the Δ*epr* and hence had jettisoned the *cat* gene and the *E. coli* replicon, a colony was selected and grown overnight in rich medium without drug selection. Individual colonies were then screened for drug resistance, and about 0.1% were found to be Cm^s. One such strain (GP216) was selected for further study.

First, Southern hybridization was used to confirm that the strain did indeed contain a deletion in the chromosomal *epr* gene (Fig. 6). Second, like the Cm^r parent strain, GP216 failed to produce a halo on casein plates (Fig. 7). In liquid cultures, however, [^{14}C]casein protease assays indicated that *epr* alone did not contribute significantly to the residual protease activity. A strain with deletion mutations in *epr*, *apr*, *npr*, and *isp* did not produce significantly less protease than a strain with mutations in just *apr*, *npr*, and *isp* in liquid culture. Apparently, this result is not reflected on a casein plate. Finally, both growth and sporulation were checked in standard laboratory media. No differences were seen in growth in LB medium. Similarly, no appreciable differences

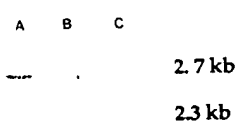

FIG. 6. Southern hybridization depicting deletion of the chromosomal *epr* gene. *B. subtilis* chromosomal DNA was digested with *Hin*dIII and probed with the nick-translated *epr* gene. (A) GP208; (B) GP208; (C) GP216 (Δ*epr*).

FIG. 7. Assay for protease activity on casein plates. A top layer containing 1% dry milk was added to tryptose blood agar base plates. (A) GP216 (Δ*epr*); (B) GP208.

were seen in sporulation frequency after growth on DSM medium (18) for 30 h (10^8 spores per ml for both GP208 and GP216).

DISCUSSION

The primary product of the *epr* gene is a 69,702-dalton protein that appears to be processed to yield a 34,000-dalton protease. Subclones of the gene encoding only the first 405 of the 640 amino acids produced the active 34,000-dalton protease, arguing that the enzyme normally undergoes C-terminal processing and that the final 240 amino acids are not required for activity. Several unusual features were observed in this C-terminal portion of the protein. First, the C-terminal domain was unusually rich in basic amino acids, with lysine accounting for approximately 24% of the amino acids. Second, a partially homologous 44-amino-acid sequence was repeated five times between nucleotides 1168 and 1927 (Fig. 2).

Two other extracellular proteases have been described which contain a large C-terminal domain that is processed. However, in both these cases (IgA protease from *Neisseria gonorrhoeae* [14] and a serine protease from *Serratia marcescens* [22]) the C-terminal domain is involved in excretion of the protease through the outer membrane of a gram-negative bacterium and is essential for enzyme production. The role of the C-terminal 240 amino acids of Epr remains to be elucidated.

The protease encoded by *epr* is a serine protease with homology (40%) to both subtilisin and ISP-1 (Fig. 3A and B). Epr does not appear to correspond to a previously described esterase, bacillopeptidase F (12, 16). The amino acid composition predicted for Epr does not resemble that deduced for bacillopeptidase F (16), and a strain carrying a deletion in the *epr* gene (GP216) did not produce less esterase activity than the parent strain GP208. R. Bruckner and R. Doi (personal communication) have independently identified a new protease gene that maps in the *sacA* region of the *B. subtilis* chromosome and is likely to be the same as *epr*.

The presence of residual protease activity in the culture fluids in *B. subtilis* strains deleted for the *epr* gene (as well as the *apr*, *npr*, and *isp* genes) suggests that *B. subtilis* contains additional genes encoding minor extracellular proteases, perhaps similar to *epr*. The extracellular protease genes characterized to date (*epr*, *apr*, and *npr*) are all located at different loci on the *B. subtilis* chromosome, and none are essential for growth or sporulation (19, 23). *apr* and *npr* are both regulated by the *sacQ*, *sacU*, *hpr*, *prtR*, and certain *spo0* genes (5, 8). It will be interesting to determine whether the *epr* gene is also regulated by these loci in the same manner as the *apr* and *npr* genes.

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Cloning of the Neutral Protease Gene of *Bacillus subtilis* and the Use of the Cloned Gene to Create an In Vitro-Derived Deletion Mutation

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The neutral protease gene of *Bacillus subtilis* has been cloned, and its nucleotide sequence has been determined. The cloned gene was used to create an in vitro-derived deletion mutation, which was used to replace the wild-type copy of the gene. This deletion, in combination with a deletion of the alkaline protease gene, completely abolished protease production. The loss of the proteases had no detectable effect on growth, morphology, or sporulation.

Bacillus subtilis produces two major extracellular proteases, a neutral protease and an alkaline protease (or subtilisin). Both are produced after the exponential growth phase, when the culture enters the stationary phase and begins the process of sporulation. The physiological role of these two proteases is not clear. They have been postulated to play a role in sporulation (15, 35, 36), to be involved in the regulation of cell wall turnover (17), and to be scavenger enzymes (36). The regulation of expression of the protease genes appears to be complex. They appear to be coordinately regulated with sporulation, since mutants blocked in the early stages of sporulation exhibit reduced levels of both the alkaline and neutral proteases (15, 35). Additionally, a number of pleiotropic mutations exist that affect the level of expression of proteases and other secreted gene products, such as amylase and levansucrase (36). The neutral protease structural gene also has a tightly linked regulatory gene, *nprR2*, which overproduces neutral protease about 30-fold as compared with the wild-type allele, *nprR1* (44).

Well-characterized mutations of these protease genes have been difficult to obtain for a number of reasons. Since there exist at least two proteases, screening for the loss of one is difficult. Additionally, the large number of pleiotropic mutations affecting both sporulation and protease production make the isolation of true protease mutations difficult. Temperature-sensitive mutants of the neutral protease gene have been obtained and were used to map the position of the structural gene, *nprE* (43). Additionally, a presumed nonsense mutation of the alkaline protease gene was characterized, but never mapped (39).

To facilitate the study of the regulation of these protease genes and their physiological role(s), we decided to clone the structural genes for the two major proteases. Such an approach should allow a characterization of the regulatory regions involved in their expression. Additionally, one might then create precisely defined mutations of the genes and study their effects upon transfer back into the genome. A previous report detailed the cloning and creation of a deletion mutant of the alkaline protease gene (42). This report details the cloning of the neutral protease gene of *B. subtilis*, the determination of its nucleotide sequence, the creation of an in vitro-derived deletion mutation, and the effects of the loss of both the alkaline and neutral proteases.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* MM294 (F⁻ *supE44 endA1 thi-1 hsdR4*) was used for all plasmid constructions, unless otherwise indicated (2). The *B. subtilis* strains used in this study are listed in Table 1. Plasmids pJH101, pBS42, and pBR325 have been previously described (3, 8, 12). *E. coli* was transformed by the calcium chloride shock method as described previously, with selection on LB plates containing ampicillin at 20 μg/ml or chloramphenicol at 12.5 μg/ml (14). *B. subtilis* strains were transformed by the procedure of Anagnostopoulos and Spizizen (1); 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid was added to the stage II media for plasmid transformations (13). Selection was on tryptose-blood agar base (Difco Laboratories) or LB skim milk plates (1.5% Carnation powdered nonfat milk) supplemented with chloramphenicol (5 μg/ml) for plasmid transformation. Auxotrophic markers were selected on minimal glucose plates supplemented with the appropriate amino acids (50 μg/ml) (41) or on C medium plus fructose (34). The preparation of PBS1 transducing lysates and PBS1 transduction was as previously described (16).

Reagents. Restriction enzymes, polynucleotide kinase, bacterial alkaline phosphatase, and the Klenow fragment of DNA polymerase I were purchased from commercial sources and used according to the suppliers' conditions. A packaging mix was purchased from a commercial source (Packagene; Promega Biotec). Oligonucleotides were provided by the Genentech Organic Synthesis Group and were synthesized by the phosphotriester method (11).

Phage and plasmid constructions. A Charon 30 bank of *B. subtilis* GSY264 DNA was constructed essentially as previously described (47). Partial *Sau*3A fragments were sized on an agarose gel, and 15 to 20-kilobase fragments were eluted, ligated into Charon 30 arms, packaged, and plated with strain DP50 *supF* as a host (7). The separation of restriction fragments on polyacrylamide and agarose gels and the electroelution of DNA fragments were performed as previously described (19). All plasmid constructions were made with DNA purified by electroelution from gels. Chromosomal, phage, and plasmid DNAs were prepared as previously described (4, 6, 7, 10, 22). DNA sequencing was performed by the chain-terminating dideoxy method (38).

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype[a]	Origin
BG16	*purB6 metB5 leuA8 lys-21 hisA thr-5 sacA321*	A. Galizzi (PB1665)
BG40	*argC metC ura*	A. Galizzi (PB1749)
PG524	*fruB22 ura-3*	BGSC (1A237)
BG84	*Δspo0A677 prt-77*	(42)
GSY264	*mtr-264*	BGSC (1A72)
BG2018	*trpC2 prt-77 Δapr-677*	(42)
BG2019	*Δapr-684*	(42)
BG2034	*Δapr-684 npr*::pNPRsubH1Δ	pNPRsubH1Δ × BG2019
BG2035	*Δapr-684 purB6 leuA8 lys-21 thr-5 hisA*	E. Ferrari
BG2036	*Δapr-684 ΔnprE522*	Cm[s] of BG2034
BG2043	*ΔnprE-522 leuA8 thr-5 hisA (sacA321)*	BG2036 DNA × BG16
BG2054	*ΔnprE-522 leuA8 thr-5 Δapr-684 (sacA321)*	BG2018 DNA × BG2043

[a] The presence of loci shown within parentheses was not tested.

For certain fragments dITP was used to reduce compression artifacts (28). Specific restriction fragments were ligated into the appropriate M13 vectors mp8 or mp9 (27).

Filter hybridizations. Transfer of DNA from agarose gels to nitrocellulose filters was by the method of Southern (40) after depurination (45). Screening of phage plaques by transfer to nitrocellulose was done as described previously (5). Oligonucleotides were labeled by phosphorylation with [γ-^{32}P]ATP (~5,000 Ci/mmol; New England Nuclear Corp.). DNA fragments were labeled with [α-^{32}P]dCTP (~3,000 Ci/mmol; New England Nuclear) by nick translation. Hybridization and washing conditions for fragments labeled by nick translation were as previously described (24). Hybridizations to phosphorylated oligonucleotide probes were at 37°C in a solution of 5× Denhardt solution, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM $NaPO_4$ (pH 6.8), and 20% formamide. Filters were prehybridized in the same buffer also containing 250 μg of sheared denatured salmon sperm DNA per ml. Washing was in 6× SSC at 37°C.

Assays. Protease assays were done by adding 0.1 ml of the culture supernatant to a suspension of 1.4 mg of azocoll (Sigma Chemical Co.) per ml in 10 mM Tris-hydrochloride–100 mM NaCl (pH 6.8) and incubating with agitation at 37°C for various times. Undigested azocoll was pelleted by centrifugation, and the optical density at 505 nm was determined. Background values of an azocoll suspension without supernatant were subtracted. Cultures for protease assays were grown for 16 h in minimal media supplemented with 0.1% casein hydrolysate and all auxotrophic requirements. Chloramphenicol (10 μg/ml) was added to the media of strains carrying plasmids. Sporulation was determined by growing cultures in LDSM sporulation media (20) supplemented with auxotrophic requirements and chloramphenicol (10 μg/ml) for strains carrying plasmids. The total cell count was determined by plating appropriate dilutions of the culture, and the number of spores was determined by plating dilutions after heating to 80°C for 10 min. The percent sporulation was the number of spores divided by the total cell count. In vitro transcription-translation assays were done with a kit (Amersham Corp.). The reaction products were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (23). After electrophoresis, the gel was impregnated with Enlighten (New England Nuclear) and fluorographed.

RESULTS

Design of the oligonucleotide probe. The 17-mer oligonucleotide probes for the neutral protease gene were based on the published partial amino acid sequence of the *B. subtilis* neutral protease (21). The rationale is outlined in Fig. 1, where the six amino acids used and all of the possible codons are shown. Twenty-four combinations are necessary to cover all of the possible coding sequences. Four pools of six 17-mer oligonucleotides were synthesized (Fig. 1). No other region of the published protein sequence could be covered with such a limited set of oligonucleotides.

Genomic hybridizations. To determine whether the sequences of the oligonucleotide probes were unique in the *B. subtilis* genome, and to empirically determine the proper hybridization conditions, each of the oligonucleotide pools was labeled by phosphorylation with [γ-^{32}P]ATP and hybridized to a blot of *B. subtilis* GSY264 DNA that had been digested with various restriction enzymes. Hybridizations were performed at increasing stringency until a single band was seen to hybridize with the probe. At those conditions (as detailed above), pool 4 hybridized strongly to an *Eco*RI band of approximately 4,300 base pairs (bp) and to an *Hin*dIII band of approximately 2,400 bp. Pools 2 and 3 hybridized weakly to the same bands, whereas pool 1 showed no hybridization under those conditions. Thus pool 4 was chosen as the probe for the isolation of the gene.

Isolation of a λ phage containing the neutral protease gene. A λ library of strain GSY264 DNA was screened by hybridization with labeled oligonucleotide pool 4, and several positive plaques were identified. Each of the positive plaques was purified through two rounds of single-plaque purification, and two plaques were chosen for further study, designated λ NPRG1 and λ NPRG2. DNA was prepared from each phage, and DNA blot hybridization analysis showed that λ NPRG1 contained a 2,400-bp *Hin*dIII fragment that hybridized to oligonucleotide pool 4, but not a 4,300-bp *Eco*RI fragment, whereas λ NPRG2 contained a 4,300-bp *Eco*RI fragment, but not a 2,400-bp *Hin*dIII fragment. The 2,400-bp *Hin*dIII fragment of λ NPRG1 was subcloned into the *Hin*dIII site of pJH101. Several plasmids were isolated that contained the 2,400-bp fragment. Restriction analysis showed that both orientations of the fragment were obtained; one orientation was designated pNPRsubH1, and the other orientation was designated pNPRsubHb. The 4,300-bp *Eco*RI fragment of λ NPRG2 was subcloned into the *Eco*RI site of pBR325. Again, both orientations of the fragment were obtained; these were designated pNPRsubRI and pNPRsubRIb.

Characterization of the neutral protease gene. A restriction map of the insert in pNPRsubH1 was obtained by standard restriction analysis (Fig. 2). DNA blot hybridization was used to localize the region of the presumed coding sequence of the neutral protease gene. A 430-bp *Rsa*I fragment was the smallest fragment that hybridized to the probe, and that

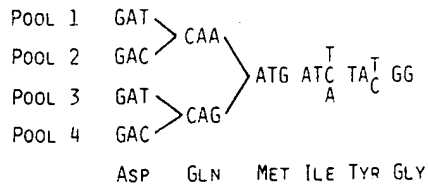

FIG. 1. Sequence of oligonucleotide probes.

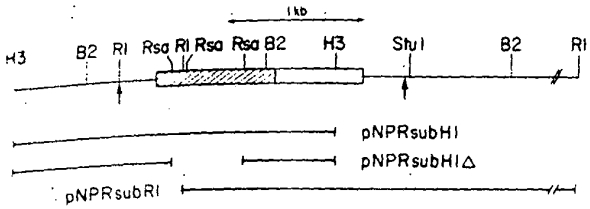

FIG. 2. Restriction map of the neutral protease gene. This restriction map is a composite of sequence data, DNA blot hybridizations, and restriction analysis of cloned DNA fragments. Only sites discussed in the text are noted. Arrows indicate the ends of the sequenced region. Double lines indicate the coding region for the neutral protease gene, and filled double lines indicate the coding region for the mature protein. Single lines below the restriction map indicate the extent of the DNA carried by various plasmid clones. Transcription of the gene is from right to left. Abbreviations: H3, *Hin*dIII; B2, *Bgl*II; RI, *Eco*RI; *Rsa*, *Rsa*I.

fragment was ligated in the *Sma*I site of M13 mp8. The sequence of the *Rsa*I fragment was determined, and a sequence that matched that of the oligonucleotide probe was found. Translation of the surrounding sequence matched almost exactly the published protein sequence of the neutral protease (21). Other restriction fragments from the pNPRsubH1 insert were ligated into appropriate M13 vectors and sequenced. An open reading frame spanning the published amino and carboxyl terminus of the neutral protease was determined. The open reading frame extended past the published amino terminus to the end of the cloned *Hin*dIII fragment. The 1,300-bp *Bgl*II-*Hin*dIII fragment from pNPRsubRIb was cloned into M13 mp8, and the sequence was determined for an additional 400 nucleotides from the *Hin*dIII site (Fig. 2). The nucleotide sequence and the translated amino acid sequence are shown in Fig. 3.

Genetic mapping of the neutral protease gene. The chromosomal location of the neutral protease gene has been previously determined to lie between the *fruB* and *cysC* loci (43). The genetic location of the cloned 2,400-bp *Hin*dIII fragment of pNPRsubH1 was determined by transformation of the pNPRsubH1 into strain I168 and selection for integration of the plasmid by selection for Cm^r. PBS1 transducing lysates of the strain carrying the integrated plasmids were prepared and used to transduce strain BG40 (*metC ura*) and PG524 (*ura-3 fruB22*) with selection for Cm^r. Linkage of the *fruB*, *metC*, and *ura* loci to the Cm^r were 80, 18, and 45%, respectively. Analysis of the transductants from three-factor crosses suggested that the gene order is *metC-fruB*-Cm^r-*ura*. These data are consistent with the previously determined location of the neutral protease gene.

Creation of a deletion mutation of the neutral protease gene. To create a deletion mutation of the neutral protease gene, a plasmid was constructed that was deleted for the two *Rsa*I fragments in the insert of pNPRsubH1. The deletion of the two *Rsa*I fragments deletes 527 nucleotides from the coding sequence of the neutral protease gene. pJH101 was digested with *Hin*dIII, treated with bacterial alkaline phosphatase, and ligated with the left-hand 1,200-bp *Hin*dIII-*Rsa*I and right-hand 680-bp *Rsa*I-*Hin*dIII fragments of the insert of pNPRsubH1 (Fig. 2). A plasmid was isolated which had the left- and right-hand fragments in the same orientation as in pNPRsubH1, but which lacked the internal *Rsa*I fragments. This plasmid was designated pNPRsubH1Δ.

Replacement of the wild-type neutral protease gene with a deletion mutation. Plasmid pNPRsubH1Δ was transformed into *B. subtilis* BG2019 and integrants were selected on LB skim milk plates plus chloramphenicol. Two types of Cm^r transformants were noted, those with parental levels of proteolysis surrounding the colony and those with almost no zone of proteolysis. Those lacking a zone of proteolysis were picked and restreaked to purify individual colonies, and their protease-deficient character on skim milk plates was confirmed. One of the Cm^r, proteolysis-deficient colonies was chosen for further studies (designated BG2034). Spontaneous Cm^s revertants of BG2034 were isolated by overnight growth in LB medium containing no chloramphenicol, plating for individual colonies, and replica plating on media with and without chloramphenicol. Three Cm^s revertants were isolated, two of which were protease proficient and one of which had the protease-deficient phenotype of the parent (designated BG2036). DNA blot hybridization analysis was used to confirm the genotypes of BG2034. Figure 4 shows such a blot of *Hin*dIII-digested DNA. Lane 1 shows the wild-type pattern, with an *Hin*dIII band of 2,400 bp; lane 2 shows a strain carrying the integrated plasmid pNPRsubH1Δ, and the wild-type 2,400-bp fragment is detected along with the 1,900-bp deletion fragment; lane 3 shows the strain from which the plasmid was lost, leaving behind the 1,900-bp deletion fragment. Lanes 4 and 5 show *Hin*dIII digests of pNPRsubH1 and pNPRsubH1Δ, respectively.

Expression of the neutral protease gene on a high-copy plasmid. The neutral protease gene was placed on a high-copy plasmid to determine whether the neutral protease would be overexpressed. Figure 5 shows the construction of a plasmid, pNPR10, carrying the structural gene for the neutral protease and approximately 900 bp of sequence past the amino terminus of the gene, which should contain the regulatory signals for expression. Strain BG84 carrying pNPR10 overexpressed neutral protease as determined on milk plates (Fig. 6) and by measurement of protease activity in culture supernatants (Table 2). Plasmid pNPR10 was also used to program an in vitro transcription-translation assay to confirm the size of the coding region of the gene determined by the sequence. Figure 7 shows the translation products of pNPR10 and the parent plasmid pBS42. A protein migrating with an apparent molecular weight of 67,000 is seen in the assay programmed with pNPR10 (Fig. 7, lanes 1 through 3) that is not present in the assay programmed with pBS42 (lane 5). Although the apparent molecular weight is significantly larger than the translated molecular weight of the neutral protease gene (55,000), the mature neutral protease migrates on gels with an apparent molecular weight of 45,000, although its actual molecular weight is 33,000 (48).

Phenotype of strains lacking functional proteases and overexpressing proteases. The growth, sporulation, and expres-

TABLE 2. Effect of protease deletions on protease expression and sporulation

Strain	Genotype[a]	Protease activity[b]	% Sporulation
BG16	Wild type	100	40
BG2035	*Δapr-684*	70	20
BG2043	*ΔnprE522*	20	20
BG2054	*Δapr-684 ΔnprE522*	ND	45
BG84(pBS42)	*Δspo0A677 prt-77*	ND	
BG84(pNPR10)	*Δspo0A677 prt-77*	3,000	

[a] Only the loci relevant to the protease phenotype are shown. Complete genotypes are shown in Table 1.

[b] Protease activity is expressed in arbitrary units. BG16 was assigned a level of 100. ND indicates that the level of protease was not detectable in the assay used.

sion of proteases was examined in strains lacking a functional gene for either the neutral or alkaline protease or both. The expression of proteases was examined by a zone of clearing surrounding a colony on a skim milk plate (Fig. 6) and by measurement of the protease levels in liquid culture supernatants (Table 2). Strain BG2035, carrying a deletion of the alkaline protease gene, showed a 30% reduction in the level of protease activity and a normal halo on milk plates. Strain BG2043, carrying a deletion of the neutral protease gene, showed an 80% reduction in the protease activity and only a small halo on the milk plate. Strain BG2054, carrying deletions in both genes, showed no detectable protease activity in this assay and no detectable halo on milk plates. The deletion of either or both of the protease genes had no apparent effect on either growth or sporulation. Strains carrying these deletions had no detectable alteration in growth rates (data not shown). The strains sporulated at frequencies comparable to that of the parent strain BG16 (Table 2). In other genetic backgrounds, strains carrying both protease deletions, for example, BG2036, showed sporulation frequencies of >90%. Examination of the morphology of these strains showed no apparent differences from strains without such deletions. Additionally, the morphology of strains overproducing proteases on a high-copy plasmid was examined. Strain BG2036 was transformed with either pBS42, pNPR10, or pS4, a plasmid carrying the *Bacillus*

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CACATGACAC TTGACTCATC TTGATATTAT TCAACAAAAA CAAACACAGG ACAATACTAT CAATTTTGTC TAGTTATGTT AGTTTTTGTT GAGTATTCCA GAATGCTAGT TTAATATAAC AATATAAAGT TTTCAGTATT

                                1                                  10                                  20                                  30
                           met gly leu gly lys lys leu ser val arg val ala ala ser phe met ser leu ser ile ser leu pro gly val gln ala ala glu gly his gln leu lys
TTCAAAAAGG GGGATTTATT      GTG GGT TTA GGT AAG AAA TTG TCT GTT CGT GTC GCT GCT TCG TTT ATG AGT TTA TCA ATC AGC CTG CCA GGT GTT CAG GCT GCT GAA GGT CAT CAG CTT AAA

                   40                                  50                                  60                                  70
glu asn gln thr asn phe leu ser lys lys pro ile ala gln ser glu leu ser ala pro asn asp lys ala val lys gln phe leu lys lys asn ser asn ile phe lys gly asp pro
GAG AAT CAA ACA AAT TTC CTC TCC AAA AAG CCG ATT GCG CAA TCA GAA CTC TCT GCA CCA AAT GAC AAG GCT GTC AAG CAG TTT TTG AAA AAG AAC AGC AAC ATT TTT AAA GGT GAC CCT

                   80                                  90                                 100                                 110
ser lys ser val lys leu val glu ser thr thr asp ala leu gly tyr lys his phe arg tyr ala pro val val asn gly val pro ile lys asp ser gln val ile val his val asp
TCC AAA AGC GTG AAG CTT GTT GAA AGC ACG ACT GAT GCC CTT GGA TAC AAG CAC TTT CGA TAT GCG CCT GTC GTT AAC GGA GTG CCA ATT AAA GAT TCG CAA GTG ATC GTT CAC GTC GAT

                  120                                 130                                 140                                 150
lys ser asp asn val tyr ala val asn gly glu leu his asn gln ser ala ala lys thr asp asn ser gln lys val ser ser glu lys ala leu ala leu ala phe lys ala ile gly
AAA TCC GAT AAT GTC TAT GCG GTC AAT GGT GAA TTA CAC AAT CAA TCT GCT GCA AAA ACA GAT AAC AGC CAA AAA GTC TCT TCT GAA AAA GCG CTG GCA CTC GCT TTC AAA GCT ATC GGC

                  160                                 170                                 180                                 190
lys ser pro asp ala val ser asn gly ala ala lys asn ser asn lys ala glu leu lys ala ile glu thr lys asp gly ser tyr arg leu ala tyr asp val thr ile arg tyr val
AAA TCA CCA GAC GCT GTT TCT AAC GGA GCG GCC AAA AAC AGC AAT AAA GCC GAA TTA AAA GCG ATA GAA ACA AAA GAC GGC AGC TAT CGT CTT GCT TAC GAC GTG ACG ATT CGC TAT GTC

                  200                                 210                                 220    Mature                       230
glu pro glu pro ala asn trp glu val leu val asp ala glu thr gly ser ile leu lys gln gln asn lys val glu his ala ala ala thr gly ser gly thr thr leu lys gly ala
GAG CCT GAA CCT GCA AAC TGG GAA GTC TTA GTT GAC GCC GAA ACA GGC AGC ATT TTA AAA CAG CAA AAT AAA GTA GAA CAT GCC GCC GCC ACT GGA AGC GGA ACA ACG CTA AAG GGC GCA

                  240                                 250                                 260                                 270
thr val pro leu asn ile ser tyr glu gly gly lys tyr val leu arg asp leu ser lys pro thr gly thr gln ile ile thr tyr asp leu gln asn arg gln ser arg leu pro gly
ACT GTT CCT TTG AAC ATC TCT TAT GAA GGC GGA AAA TAT GTT CTA AGA GAT CTT TCA AAA CCA ACA GGC ACC CAA ATC ATC ACA TAT GAT TTG CAA AAC AGA CAA AGC CGC CTT CCG GGC

                  280                                 290                                 300                                 310
thr leu val ser ser thr thr lys thr phe thr ser ser ser gln arg ala ala val asp ala his tyr asn leu gly lys val tyr asp tyr phe tyr ser asn phe lys arg asn ser
ACG CTT GTC TCA AGC ACA ACG AAA ACA TTT ACA TCT TCA TCA CAG CGG GCA GCC GTT GAC GCA CAC TAT AAC CTC GGT AAA GTG TAC GAT TAT TTT TAT TCA AAC TTT AAA CGA AAC AGC

                  320                                 330                                 340                                 350
tyr asp asn lys gly ser lys ile val ser ser val his tyr gly thr gln tyr asn asn ala ala trp thr gly asp gln met ile tyr gly asp gly asp gly ser phe phe ser pro
TAT GAT AAC AAA GGC AGT AAA ATC GTT TCT TCC GTT CAC TAC GGC ACT CAA TAC AAT AAC GCT GCA TGG ACA GGA GAC CAG ATG ATT TAC GGT GAT GGC GAC GGT TCA TTC TTC TCT CCG

                  360                                 370                                 380                                 390
leu ser gly ser leu asp val thr ala his glu met thr his gly val thr gln glu thr ala asn leu ile tyr glu asn gln pro gly ala leu asn glu ser phe ser asp val phe
CTT TCC GGC TCA TTA GAT GTG ACA GCG CAT GAA ATG ACA CAT GGC GTC ACC CAA GAA ACA GCC AAC TTG ATT TAT GAA AAT CAG CCA GGT GCA TTA AAC GAG TCT TTC TCT GAC GTA TTC

                  400                                 410                                 420                                 430
gly tyr phe asn asp thr glu asp trp asp ile gly glu asp ile thr val ser gln pro ala leu arg ser leu ser asn pro thr lys tyr asn gln pro asp asn tyr ala asn tyr
GGG TAT TTT AAC GAT ACA GAA GAC TGG GAC ATC GGT GAA GAC ATT ACG GTC AGC CAG CCT GCT CTT CGC AGC CTG TCC AAC CCT ACA AAA TAC AAC CAG CCT GAC AAT TAC GCC AAT TAC

                  440                                 450                                 460                                 470
arg asn leu pro asn thr asp glu gly asp tyr gly gly val his thr asn ser gly ile pro asn lys ala ala tyr asn thr ile thr lys leu gly val ser lys ser gln gln ile
CGA AAC CTT CCA AAC ACA GAT GAA GGC GAT TAT GGC GGT GTA CAC ACA AAC AGC GGA ATT CCA AAC AAA GCC GCT TAC AAC ACC ATC ACA AAA CTT GGT GTA TCT AAA TCA CAG CAA ATC

                  480                                 490                                 500                                 510
tyr tyr arg ala leu thr thr tyr leu thr pro ser ser thr phe lys asp ala lys ala ala leu ile gln ser ala arg asp leu tyr gly ser thr asp ala ala lys val glu ala
TAT TAC CGT GCG TTA ACA ACG TAC CTC ACG CCT TCT TCC ACG TTC AAA GAT GCC AAG GCA GCT CTC ATT CAG TCT GCC CGT GAC CTC TAC GGC TCA ACT GAT GCC GCT AAA GTT GAA GCA

                  520 521
ala trp asn ala val gly leu OC
GCC TGG AAT GCT GTT GGA TTG TAA TATTAGGAAA AGCCTGAGAT CCCTCAGGCT TTTATTGTTA CATATCTTGA TTTCTCTCTC AGCTGAAACG ACGAAAAGAT GCTGCCATGA GACAGAAAAC CGCTCCTGAT

TTGCATAAAG AGGGATGCAG CCGCAAGTGC GCATTTTATA AAAGCTAATG ATTCAGTCCA CATAATTGAT AGACGAATTC
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FIG. 3. Nucleotide sequence of the neutral protease gene. The amino acid sequence of the predicted polypeptide product is shown above the nucleotide sequence. Numbers above the line refer the amino acid positions. The start site of the mature product is amino acid 222. The potential Shine-Dalgarno sequence is underlined, and the position of the inverted repeat sequence following the gene is indicated by overlining.

FIG. 4. DNA blot hybridization analysis of the neutral protease gene. Shown are the electrophoretic patterns of *Hin*dIII-digested DNA of strain BG2019 (lane 1), strain BG2034 (lane 2), strain BG2036 (lane 3), plasmid pNPRsubH1 (lane 4), and plasmid pNPRsubH1Δ (lane 5). The probe used was the 2,400-bp *Hin*dIII fragment of pNPRsubH1. The figure is a composite of two exposures of the same filter to compensate for the increased signal in lanes 4 and 5.

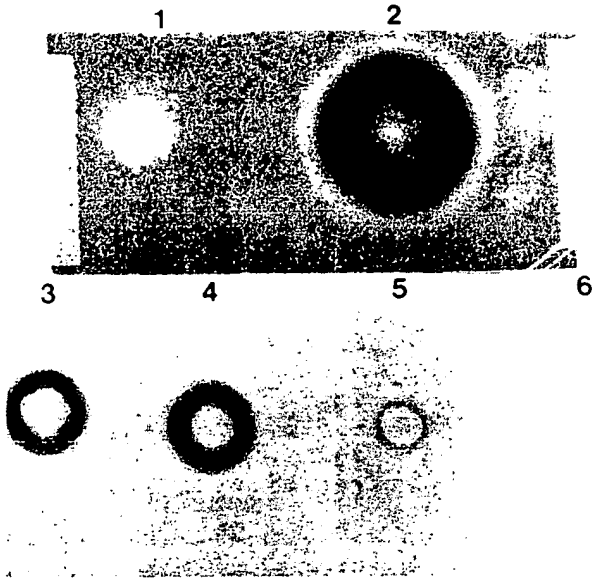

FIG. 6. Assay for protease activity on milk plates. Bacterial cells were patched on milk plates and incubated for 16 h at 37°C. The presence of a clearing zone indicates protease activity. The colonies depicted are as follows: 1, BG84(pBS42); 2, BG84(pNPR10); 3, BG16; 4, BG2035; 5, BG2043; 6, BG2054.

amyloliquifaciens subtilisin gene (46), and examined during both the exponential and stationary phases. No morphological difference in cell shape or chain formation could be detected between those cultures that lacked protease production [BG2036(pBS42)], overproduced neutral protease [BG2036(pNPR10)], or overproduced subtilisin [BG2036(pS4)].

DISCUSSION

Several features of the sequence of the neutral protease gene are of interest. The initiation codon is a GTG, which appears to be a common initiation codon in *B. subtilis* (29). It is preceded by a potential Shine-Dalgarno sequence AAAGGGGGAT, which could form a stable interaction with the 3′ end of 16S rRNA, with a ΔG of −21.6 kcal (ca. −90.44 kJ). This is similar to Shine-Dalgarno regions seen for other genes from gram-positive organisms (26, 29, 30). Immediately following the end of the coding sequence is a potential hairpin structure, with a stem of 11 bp, a loop of 4 bp, and a potential ΔG of −19.6 kcal (ca. −82.07 kJ). This is again similar to structures seen following other genes in gram-positive organisms and which are presumed to be terminators; however, functional evidence for any such role is still lacking (42, 46, 47).

The deduced amino acid sequence of the mature portion of the neutral protease matches the published amino acid sequence in 146 of 171 reported residues (21). The reason for the relatively large number of mismatches is unclear; however, the amino acid sequence was of an enzyme isolated from a strain of *B. subtilis* used for commercial production (NRRLB3411). In the past, many commercial strains were designated *B. subtilis*, when they were actually other species of *Bacillus*. It is possible that the reported protein sequence is for the neutral protease of another organism related to *B. subtilis*. The most interesting feature of the neutral protease sequence is that there appear to be 222 amino acids preced-

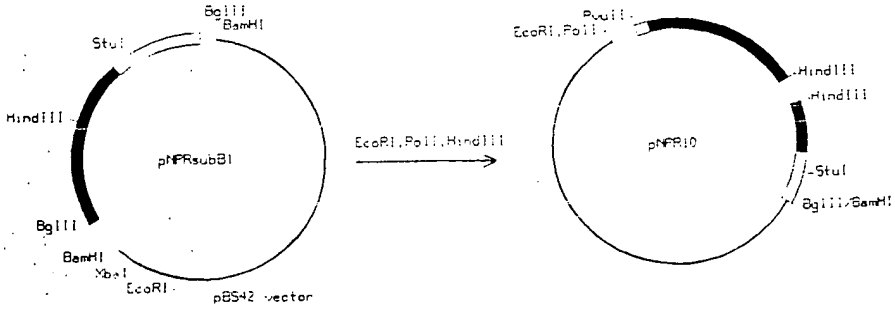

FIG. 5. Construction of a plasmid carrying the neutral protease gene. Plasmid pBS42 was digested with *Bam*HI and treated with bacterial alkaline phosphatase. The 1,900-bp *Bgl*II fragment of pNPRsubR1, containing the amino-terminal portion of the neutral protease gene, was ligated into the *Bam*HI site of pBS42 and transformed into strain MM294, and transformants were selected on LB-chloramphenicol plates. A plasmid with the *Bgl*II fragment in the indicated orientation was isolated and designated pNPRsubB1. pNPRsubB1 was digested with *Eco*RI, repaired to flush ends with the Klenow fragment of *Pol*I, and digested with *Hin*dIII. The 1,400-bp *Pvu*II-*Hin*dIII fragment of pNPRsubH1, containing the carboxyl portion of the neutral protease gene, was ligated to the pNPRsubB1 fragment and transformed into *B. subtilis* BG84, and transformants were selected on skim milk-chloramphenicol plates. Plasmids from colonies that cleared a large halo were analyzed and shown to have the indicated structure.

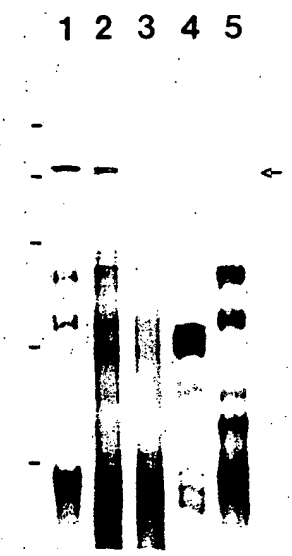

FIG. 7. In vitro transcription-translation of pNPR10. Lanes 1 through 3 show the in vitro transcription-translation products of three isolates of pNPR10, lane 4 shows the products of pAT153 (provided with the kit), and lane 5 shows the products of pBS42. An arrow indicates the position of a protein unique to pNPR10. The positions of the stained molecular mass markers are indicated; their sizes, from the top of the figure, are 92.5, 66.2, 43, 31, and 21 kilodaltons.

ing the reported amino terminus of the mature enzyme found in culture supernatant. Presumably the first 30 of these amino acids represent a signal sequence like that found for other secreted proteins (18). These amino acids appear to be a typical signal sequence, with charged amino acids (Lys-5, Lys-6, Arg-10), followed by a hydrophic core (Ala-12 through Leu-22), and a small side-chain amino acid at the cleavage point (Ala-27 or Ala-28). The additional 190 amino acids presumably represent a pro sequence, such as found for the alkaline protease genes of *B. subtilis* and *B. amyloliquifaciens* (42, 46). The pro sequences of the alkaline proteases are much shorter, only about 70 amino acids. However the pro sequences of both types of protease contain a large number of charged residues. The functional role of these pro sequences is uncertain, as is the mechanism of cleavage to the mature form of the enzyme. These cloned genes should facilitate mutational analysis of these pro sequences to elucidate their function.

This study strongly suggests that the proteases of *B. subtilis* do not have roles in either sporulation or the regulation of cell morphology. We are unable to correlate our findings with a previous report that the presence or absence of proteases could have an effect on cell morphology (17). Our work suggests that the only phenotypic effect of the deletion of the protease genes is the loss of protease activity. We would therefore suggest that these enzymes have a role only as scavengers. There does exist one other reported protease in *B. subtilis* (37); however, it has only a very limited proteolysis ability and is not detectable in an assay of proteolysis, such as the azocoll and milk plate methods used in this study.

A number of groups have recently reported the expression and secretion of heterologous gene products in *B. subtilis* (9, 31–33). Two of these reports have suggested that the presence of extracellular proteases limits the accumulation of these heterologous products due to degradation (31, 32). These defined protease deletions should be valuable in determining whether such degradation is really a factor and potentially should eliminate the problem.

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L6	52	S	L5 AND MUTANT?
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CN   Azocaseinase
CN   BAPAase
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CN   Casein endopeptidase
CN   Caseinase
CN   Corolase 7089
CN   DA 10
CN   DA 10 (enzyme)
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CN   Endopeptidase O
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CN   Protease
CN   Protease P3
CN   Protein p20 proteinase
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structural consequences of mutation at the S4 substrate binding pocket.

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*enzyme modification
*enzyme structure
amino acid substitution
article
bacillus
controlled study
crystal structure
enzyme activity
enzyme kinetics
enzyme substrate complex
hydrophobicity
nonhuman
site directed mutagenesis
X ray diffraction
***alkaline proteinase**: EC, endogenous compound
asparagine
glycine
leucine
methionine
mutant protein
phenylalanine
proline
proteinase
serine
serine proteinase: EC, endogenous compound
tryptophan
valine

RN. . . 70-47-3, 7006-34-0; (glycine) 56-40-6, 6000-43-7, 6000-44-8; (leucine) 61-90-5, 7005-03-0; (methionine) 59-51-8, 63-68-3, 7005-18-7; (phenylalanine) 3617-44-5, 63-91-2; (proline) 147-85-3, 7005-20-1; (proteinase) **9001-92-7**; (serine) 56-45-1, 6898-95-9; (serine proteinase) 37259-58-8; (tryptophan) 6912-86-3, 73-22-3; (valine) 7004-03-7, 72-18-4

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L7             1 S L6 AND PB92?

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              60   FILE ENCOMPPAT2
            7237   FILE EUROPATFULL
            3763   FILE IFIPAT
            6687   FILE INPADOC
            2873   FILE JAPIO
              71   FILE PAPERCHEM2
             102   FILE PATDD
            1399   FILE PATDPA
             361   FILE PATOSDE
            1515   FILE PATOSEP
            1551   FILE PATOSWO
           20335   FILE PCTFULL
              23   FILE PIRA
              57   FILE RAPRA
              65   FILE SYNTHLINE
              13   FILE TULSA
              10   FILE TULSA2
           21166   FILE USPATFULL
            9557   FILE WPIDS
            9557   FILE WPINDEX
L8              QUE PROTEAS?
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     FILE 'CAPLUS, DGENE, USPATFULL, PCTFULL, WPIDS, EUROPATFULL, INPADOC,
     IFIPAT, JAPIO, DPCI, PATOSWO, PATOSEP, PATDPA, CAOLD, CROPU, PATOSDE,
     PATDD, PAPERCHEM2, SYNTHLINE, ENCOMPPAT, ENCOMPPAT2, RAPRA, PIRA, TULSA,
     TULSA2' ENTERED AT 13:49:54 ON 10 MAR 2001
L9         182354 S L8
L10         33576 S L9 AND ALKAL?
L11          9977 S L10 AND BACILLUS?
L12          5879 S L11 AND MUTA?
L13           336 S L12 AND PB92?
L14           329 DUP REM L13 (7 DUPLICATES REMOVED)
L15            11 S L14 AND PY<=1990
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=> index patent

FILE 'PAPERCHEM' ACCESS NOT AUTHORIZED
COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                        16.50      22.71

INDEX 'CAOLD, CAPLUS, CROPU, DGENE, DPCI, ENCOMPPAT, ENCOMPPAT2, EUROPATFULL,
       IFIPAT, INPADOC, JAPIO, PAPERCHEM2, PATDD, PATDPA, PATOSDE, PATOSEP,
       PATOSWO, PCTFULL, PIRA, RAPRA, SYNTHLINE, TULSA, TULSA2, USPATFULL,
       WPIDS, WPINDEX' ENTERED AT 13:49:35 ON 10 MAR 2001

26 FILES IN THE FILE LIST IN STNINDEX

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search error messages that display as 0* with SET DETAIL OFF.

=> s proteas?

       1041   FILE CAOLD
      74088   FILE CAPLUS
        405   FILE CROPU
      27131   FILE DGENE
       2784   FILE DPCI
         60   FILE ENCOMPPAT
         60   FILE ENCOMPPAT2
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       2873   FILE JAPIO
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         10   FILE TULSA2
      21166   FILE USPATFULL
       9557   FILE WPIDS
       9557   FILE WPINDEX

  26 FILES HAVE ONE OR MORE ANSWERS,   26 FILES SEARCHED IN STNINDEX

L8   QUE PROTEAS?

=> file hit

COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.45      23.16

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=> s l8

L9      182354 L8

=> s l9 and alkal?

  13 FILES SEARCHED...
L10      33576 L9 AND ALKAL?

=> s l10 and bacillus?

L11       9977 L10 AND BACILLUS?

=> s l11 and muta?

  23 FILES SEARCHED...
L12       5879 L11 AND MUTA?

=> s l12 and pb92?

L13        336 L12 AND PB92?

=> dup rem l13

DUPLICATE IS NOT AVAILABLE IN 'DGENE, DPCI, CAOLD, SYNTHLINE'.
ANSWERS FROM THESE FILES WILL BE CONSIDERED UNIQUE
PROCESSING COMPLETED FOR L13
L14         329 DUP REM L13 (7 DUPLICATES REMOVED)

=> s l14 and py<=1990

   5 FILES SEARCHED...
   7 FILES SEARCHED...
  10 FILES SEARCHED...
  14 FILES SEARCHED...
  22 FILES SEARCHED...
L15         11 L14 AND PY<=1990

=> d ibib ab 1-11


L15  ANSWER 1 OF 11  USPATFULL
ACCESSION NUMBER:       94:68700  USPATFULL
TITLE:                  PB92 serine protease muteins and
                        their use in detergents
INVENTOR(S):            van Eekelen, Christiaan A. G., Bergschenhoek,
                        Netherlands
```

Mulleners, Leonardus J. S. M., SV Rijen, Netherlands
Van Der Laan, Johannes C., Amsterdam, Netherlands
Misset, Onno, Delft, Netherlands
Cuperus, Roelck A., Amsterdam, Netherlands
Lensink, Johan H. A., Delft, Netherlands
PATENT ASSIGNEE(S): Gist-brocades N.V., Delft, Netherlands (non-U.S. corporation)

	NUMBER	DATE	
PATENT INFORMATION:	US 5336611	19940809	
	WO 8907642	19890824	
APPLICATION INFO.:	US 1989-427103	19891011	(7)
	WO 1989-NL5	19890213	
		19891011	PCT 371 date
		19891011	PCT 102(e) date

	NUMBER	DATE
PRIORITY INFORMATION:	EP 1988-200255	19880211

DOCUMENT TYPE: Utility
PRIMARY EXAMINER: Robinson, Douglas W.
ASSISTANT EXAMINER: Weber, Jon P.
LEGAL REPRESENTATIVE: Rae-Venter, Barbara
NUMBER OF CLAIMS: 8
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 8 Drawing Figure(s); 8 Drawing Page(s)
LINE COUNT: 1339
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB New proteolytic enzymes are provided exhibiting improved properties for application in detergents, especially laundry detergents. These enzymes are obtained by expression of a gene encoding a proteolytic enzyme having an amino acid sequence which differs at least in one amino acid from the wild type enzyme. Preferred enzymes are certain **mutants** derived from the serine **protease** of **Bacillus** nov. spec. **PB92**.

L15 ANSWER 2 OF 11 USPATFULL
ACCESSION NUMBER: 88:59037 USPATFULL
TITLE: Heat stable **alkaline proteases** produced by a **bacillus**
INVENTOR(S): Stellwag, Edmund J., Damascus, MD, United States
Durham, Donald R., Gaithersburg, MD, United States
Swann, Wayne E., Columbia, MD, United States
Nolf, Carol A., Silver Spring, MD, United States
Stewart, David B., Arlington, VA, United States
PATENT ASSIGNEE(S): Genex Corporation, Gaithersburg, MD, United States (U.S. corporation)

	NUMBER	DATE		
PATENT INFORMATION:	US 4771003	19880913		<--
APPLICATION INFO.:	US 1985-790256	19851022	(6)	

DOCUMENT TYPE: Utility
PRIMARY EXAMINER: Shapiro, Lionel M.
LEGAL REPRESENTATIVE: Saidman, Sterne, Kessler & Goldstein
NUMBER OF CLAIMS: 14
EXEMPLARY CLAIM: 1
LINE COUNT: 637
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Novel enzymes exhibiting proteolytic activity in **alkaline** media and stability at high temperatures and under **alkaline** conditions are produced by a novel **Bacillus** strain designated GX6638 or its **mutants** or variants. These enzymes are especially well-suited for inclusion in washing compositions. A culture of GX6638 has been deposited with the American Type Culture Collection,

Rockville, Md. as ATCC No. 53278.

L15 ANSWER 3 OF 11 USPATFULL
ACCESSION NUMBER: 88:52046 USPATFULL
TITLE: **Alkaline protease** produced by a **bacillus**
INVENTOR(S): Durham, Donald R., Gaithersburg, MD, United States
Stellwag, Edmund J., Greenville, MD, United States
McNamee, Clyde G., Gaithersburg, MD, United States
PATENT ASSIGNEE(S): Genex Corporation, Gaithersburg, MD, United States (U.S. corporation)

	NUMBER	DATE	
PATENT INFORMATION:	US 4764470	19880816	<--
APPLICATION INFO.:	US 1986-826378	19860205 (6)	

DOCUMENT TYPE: Utility
PRIMARY EXAMINER: Shapiro, Lionel M.
LEGAL REPRESENTATIVE: Saidman, Sterne, Kessler & Goldstein
NUMBER OF CLAIMS: 11
EXEMPLARY CLAIM: 1
LINE COUNT: 479
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB A novel enzyme exhibiting proteolytic activity in **alkaline** media and stability under **alkaline** conditions is produced by a novel **Bacillus** strain designated GX6644 or by its **mutants** or variants. The enzyme is well-suited for inclusion in washing compositions. A culture of GX6644 has been deposited with the American Type Culture Collection, Rockville, Maryland as ATCC No. 53441.

L15 ANSWER 4 OF 11 PCTFULL COPYRIGHT 2001 MicroPatent
ACCESSION NUMBER: 1989007642 PCTFULL
TITLE (ENGLISH): NOVEL PROTEOLYTIC ENZYMES AND THEIR USE IN DETERGENTS
TITLE (FRENCH): NOUVELLES ENZYMES PROTEOLYTIQUES ET LEUR UTILISATION DANS DES DETERGENTS
INVENTOR(S): VAN EEKELEN, Christiaan, Albertus, Gerardus; MULLENERS, Leonardus, Johannes, Sofie, Marie; VAN DER LAAN, Johannes, Cornelis; MISSET, Onno; CUPERUS, Roelck, Anneke; LENSINK, Johan, Herman, Albert
PATENT ASSIGNEE(S): GIST - BROCADES N.V.; VAN EEKELEN, Christiaan, Albertus, Gerardus; MULLENERS, Leonardus, Johannes, Sofie, Marie; VAN DER LAAN, Johannes, Cornelis; MISSET, Onno; CUPERUS, Roelck, Anneke; LENSINK, Johan, Herman, Albert
LANGUAGE OF PUBL.: English
DOCUMENT TYPE: Patent
PATENT INFORMATION:

NUMBER	KIND	DATE
WO 8907642	**A1**	**19890824**

DESIGNATED STATES: AU BG BR DK FI HU JP KR NO SU US
APPLICATION INFO.: WO 1989-NL5 19890213
PRIORITY (ORIGINAL): NL 1988-88200255.3 (EP) 19880211
ABEN New proteolytic enzymes are provided exhibiting improved properties for application in detergents, especially laundry detergents. These enzymes are obtained by expression of a gene encoding a proteolytic enzyme having an amino acid sequence which differs at least in one amino acid from the wild type enzyme. Preferred enzymes are certain **mutants** derived from the serine **protease** of **Bacillus** nov. spec. **PB92**.
ABF De nouvelles enzymes proteolytiques presentent des proprietes ameliorees pouvant etre appliquees dans des detergents, notamment dans

des detergents lessiviels. On obtient ces enzymes par l'expression d'un gene codant une enzyme proteolytique dont la sequence d'amino acides differe au moins dans un amino acide, de l'enzyme de type sauvage. Les enzymes preferees sont certains **mutants** derives de la **protease** de serine de **Bacillus** nov. spec. **PB92**.

L15 ANSWER 5 OF 11 PCTFULL COPYRIGHT 2001 MicroPatent
ACCESSION NUMBER: 1988006624 PCTFULL
TITLE (ENGLISH): MOLECULAR CLONING AND EXPRESSION OF GENES ENCODING PROTEOLYTIC ENZYMES
TITLE (FRENCH): CLONAGE ET EXPRESSION MOLECULAIRE DE GENES CODANT DES ENZYMES PROTEOLYTIQUES
INVENTOR(S): VAN EEKELEN, Christiaan, Albertus, Gerardus; VAN DER LAAN, Johannes, Cornelis; MULLENERS, Leonardus, Johannes, Sofie, Marie
PATENT ASSIGNEE(S): GIST-BROCADES N.V.
LANGUAGE OF PUBL.: English
DOCUMENT TYPE: Patent
PATENT INFORMATION:

NUMBER	KIND	DATE
WO 8806624	**A2**	**19880907**

DESIGNATED STATES: AU BG BR DK FI HU JP KR NO SU
APPLICATION INFO.: WO 1988-NL7 19880226
PRIORITY (ORIGINAL): NL 1987-87200358.7 19870227
ABEN Novel methods and novel industrial **Bacillus** strains are provided for enhanced production of serine **protease**. Plasmid constructs comprising the serine **protease** gene are introduced into a compatible host, generally a **Bacillus** already overproducing serine **protease**. Preferred host cells are those from **Bacillus** novo sp. **PB92** and the preferred serine **protease** gene also originates from **Bacillus PB92**. Integration and maintenance of the plasmid in the host cell chromosome can be achieved by including a temperature sensitive origin of replication in the plasmid construct and growing under selective conditions. Les nouveaux procedes et les nouvelles souches industrielles de **Bacillus** decrits servent a la production accrue de serine **protease**. Des composes de plasmide comprenant le gene de serine **protease** sont introduits dans une cellule hote compatible, generalement constituee par un **Bacillus**, etant deja a l'origine d'une surproduction de serine **protease**. Les cellules hotes preferees sont constituees par des cellules provenant de l'espece **PB92** de **Bacillus** novo et le gene prefere de serine **protease** provient egalement de l'espece **PB92** de **Bacillus**. L'integration et la conservation du plasmide dans le chromosome de la cellule hote peuvent etre obtenues par inclusion d'une source thermosensible de replication dans le compose a base de plasmide et par culture dans des conditions selectives.

L15 ANSWER 6 OF 11 PCTFULL COPYRIGHT 2001 MicroPatent
ACCESSION NUMBER: 1988006623 PCTFULL
TITLE (ENGLISH): STABLE GENE AMPLIFICATION IN CHROMOSOMAL DNA OF PROKARYOTIC MICROORGANISMS

TITLE (FRENCH): AMPLIFICATION DE GENE STABLE DANS L'ADN CHROMOSOMIQUE DE MICRO-
ORGANISMES PROCARYOTIQUES
INVENTOR(S): VAN EEKELEN, Christiaan, Albertus, Gerardus; VAN DER LAAN, Johannes, Cornelis; MULLENERS, Leonardus, Johannes, Sofie, Marie
PATENT ASSIGNEE(S): GIST-BROCADES N.V.
LANGUAGE OF PUBL.: English
DOCUMENT TYPE: Patent
PATENT INFORMATION:

	NUMBER	KIND	DATE
	WO 8806623	**A1**	**19880907**

DESIGNATED STATES: AU BG BR DK FI HU JP KR NO SU
APPLICATION INFO.: WO 1988-NL6 19880226
PRIORITY (ORIGINAL): NL 1987-87200356.1 19870227

ABEN Transformed prokaryotic hosts are provided comprising two or more copies of a DNA sequence stably maintained in their chromosome, said sequence comprising a gene encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences. Methods are also provided for producing said transformed host strains. Said transformed host strains are capable of increased production of the polypeptide of interest compared to host strains which already produce said polypeptide. Preferred host strains are **Bacillus** novo species **PB92** which produces a high-**alkaline** proteolytic enzyme and **Bacillus** licheniformis T5 which produce a thermostable alpha-amylase, and **mutants** and variants of said strains. Preferred polypeptide encoding genes are the **protease** encoding gene originating from **Bacillus** **PB92** and the alpha-amylase encoding gene originating from **Bacillus** licheniformis strain T5.
Des souches hotes procaryotiques transformees comprennent au moins deux copies d'une sequence d'ADN conservees sous forme stable dans leur chromosome, ladite sequence comprenant un gene codant un polypeptide desire dans lequel lesdites copies sont separees par des sequences d'ADN chromosomique endogene. Des procedes sont egalement decrits pour produire ses souches hotes transformees. Lesdites souches hotes transformees sont capables d'une production accrue du polypeptide desire par rapport a des souches hotes qui produisent deja ledit polypeptide. Les souches hotes preferees sont constituees par l'espece **PB92** de **Bacillus** novo qui produit une enzyme proteolytique tres alcaline et par la souche T5 de **Bacillus** licheniformis qui produit une alpha-amylase thermostable, ainsi que par des organismes **mutants** et variants desdites souches. Les genes preferes codant un polypeptide sont constitues par le gene de codage de **protease** provenant de l'espece **PB92** de **Bacillus** et par le gene de codage d'alpha-amylase provenant de la souche T5 de **Bacillus** licheniformis.

L15 ANSWER 7 OF 11 EUROPATFULL COPYRIGHT 2001 WILA

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 328229 EUROPATFULL EW 198933 FS OS STA B
TITLE: Novel proteolytic enzymes and their use in detergents.
Proteolytische Enzyme und ihre Verwendung in Waschmitteln.
Enzymes proteolytiques nouvelles et leur utilisation

dans les detergents.

INVENTOR(S): Van Eekelen, Christiaan Albertus Gerardus, Bachplaats 14, NL-2661 HD Bergschenhoek, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1, NL-1062 CP Amsterdam, NL;
Misset, Onno, Otterlaan 2, NL-2623 CX Delft, NL;
Cuperus, Roelck Anneke, Noorderstraat 119, NL-1017 TT Amsterdam, NL;
Lensink, Johan Herman Albert, Foulkeslaan 51, NL-2625 PZ Delft, NL

PATENT ASSIGNEE(S): GIST-BROCADES N.V., Wateringseweg 1, NL-2611 XT Delft, NL

PATENT ASSIGNEE NO: 200380

AGENT: Huygens, Arthur Victor, Dr. et al, c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1, NL-2600 MA Delft, NL

AGENT NUMBER: 20213

OTHER SOURCE: ESP1989034 EP 0328229 A1 890816

SOURCE: Wila-EPZ-1989-H33-T1

DOCUMENT TYPE: Patent

LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch

DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE

PATENT INFO.PUB.TYPE: EPA1 EUROPAEISCHE PATENTANMELDUNG

PATENT INFORMATION:

	PATENT NO	KIND	DATE
	EP 328229	**A1**	**19890816**
'OFFENLEGUNGS' DATE:			19890816
APPLICATION INFO.:	EP 1989-200350		19890213
PRIORITY APPLN. INFO.:	EP 1988-200255		19880211

ABEN New proteolytic enzymes are provided exhibiting improved properties for application in detergents, especial.shy. ly laundry detergents. These enzymes are obtained by expres.shy. sion of a gene encoding a proteolytic enzyme having an amino acid sequence which differs at least in one amino acid from the wild type enzyme. Preferred enzymes are certain **mutants** derived from the serine **protease** of **Bacillus** nov. spec. **PB92**.

L15 ANSWER 8 OF 11 EUROPATFULL COPYRIGHT 2001 WILA

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 284126 EUROPATFULL EW 198839 FS OS STA B

TITLE: Stable gene amplification in prokaryotic chromosomal DNA.
Stabile Genamplifizierung in prokaryotischer chromosonaler DNA.
Amplification de genes stable dans l'ADN chromosomique des prokaryotes.

INVENTOR(S): Van Eekelen, Christiaan Albertus Gerardus, Bachplaats 14, NL-2661 HD Bergschenhoek, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1, NL-1062 CP Amsterdam, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL

PATENT ASSIGNEE(S): GIST-BROCADES N.V., Wateringseweg 1, NL-2611 XT Delft, NL

PATENT ASSIGNEE NO: 200380
AGENT: Huygens, Arthur Victor, Dr. et al, c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1, NL-2600 MA Delft, NL
AGENT NUMBER: 20213
OTHER SOURCE: ESP1988035 EP 0284126 A1 880928
SOURCE: Wila-EPZ-1988-H39-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
PATENT INFO.PUB.TYPE: EPA1 EUROPAEISCHE PATENTANMELDUNG
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 284126	**A1**	**19880928**

'OFFENLEGUNGS' DATE: 19880928
APPLICATION INFO.: EP 1988-200376 19880229
PRIORITY APPLN. INFO.: EP 1987-200356 19870227
ABEN Transformed prokaryotic hosts are provided comprising two or more copies of a DNA sequence stably maintained in their chromosome, said sequence comprising a gene encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences. Methods are also provided for producing said transformed host strains. Said transformed host strains are capable of increased production of the polypeptide of interest compared to host strains which already produce said polypeptide. Preferred host strains are **Bacillus** novo species **PB92** which produces a high-.shy. **alkaline** proteolytic enzyme and **Bacillus** licheniformis T5 which produce a thermostable alpha-amylase, and **mutants** and variants of said strains. Preferred polypeptide encoding genes are the **protease** encoding gene originating from **Bacillus** **PB92** and the alpha-amylase encoding gene originating from **Bacillus** licheniformis strain T5.

L15 ANSWER 9 OF 11 EUROPATFULL COPYRIGHT 2001 WILA

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 283075 EUROPATFULL EW 198838 FS OS STA B
TITLE: Molecular cloning and expression of genes encoding proteolytic enzymes.
Molekulare Klonierung und Expression von proteolytische Enzyme kodierenden Genen.
Clonage moleculaire et expression de genes codant pour des enzymes proteolytiques.
INVENTOR(S): Van Eekelen, Christian Albertus Gerardus, Bachplaats 14,
NL-2661 HD Bergschenhoek, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1,
NL-1062 CP Amsterdam, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL
PATENT ASSIGNEE(S): GIST-BROCADES N.V., Wateringseweg 1, NL-2611 XT Delft, NL
PATENT ASSIGNEE NO: 200380
AGENT: Huygens, Arthur Victor, Dr. et al, c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1, NL-2600 MA Delft, NL
AGENT NUMBER: 20213
OTHER SOURCE: ESP1988034 EP 0283075 A2 880921
SOURCE: Wila-EPZ-1988-H38-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch

DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
PATENT INFO.PUB.TYPE: EPA2 EUROPAEISCHE PATENTANMELDUNG
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 283075	**A2**	**19880921**

'OFFENLEGUNGS' DATE: 19880921
APPLICATION INFO.: EP 1988-200377 19880229
PRIORITY APPLN. INFO.: EP 1987-200358 19870227

GRANTED PATENT - ERTEILTES PATENT - BREVET DELIVRE

ACCESSION NUMBER: 283075 EUROPATFULL EW 199919 FS PS
TITLE: Molecular cloning and expression of genes encoding proteolytic enzymes.
Molekulare Klonierung und Expression von proteolytische Enzyme kodierenden Genen.
Clonage moleculaire et expression de genes codant pour des enzymes proteolytiques.
INVENTOR(S): Van Eekelen, Christian Albertus Gerardus, Bachplaats 14,
NL-2661 HD Bergschenhoek, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1,
NL-1062 CP Amsterdam, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL
PATENT ASSIGNEE(S): GENENCOR INTERNATIONAL, INC., 4 Cambridge Place, 1870 South Winton Road, Rochester, New York 14618, US
PATENT ASSIGNEE NO: 1285780
AGENT: Armitage, Ian Michael et al, MEWBURN ELLIS York House 23
Kingsway, London WC2B 6HP, GB
AGENT NUMBER: 27761
OTHER SOURCE: EPB1999029 EP 0283075 B1 990512
SOURCE: Wila-EPS-1999-H19-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
DESIGNATED STATES: R AT; R BE; R DE; R ES; R FR; R GB; R IT; R NL
PATENT INFO.PUB.TYPE: EPB1 EUROPAEISCHE PATENTSCHRIFT
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 283075	**B1**	**19990512**

'OFFENLEGUNGS' DATE: 19880921
APPLICATION INFO.: EP 1988-200377 19880229
PRIORITY APPLN. INFO.: EP 1987-200358 19870227
REFERENCE PAT. INFO.: EP 108301 A EP 130756 A
EP 134048 A EP 138075 A
EP 251446 A WO 86-01825 A
WO 87-04461 A FR 2292042 A
REF. NON-PATENT-LIT.: APPL BIOCHEM MICROBIOL, vol.14, no.10, 1978/9, page 511 - 523, '' APPL MICROBIOL BIOTECHNOL, vol.34, no., 1990, page 57 - 62, '' AGRIC BIOL CHEM, vol.48, no.2, 84, page
397 - 401, '' CRIT REV BIOTECHNOL, vol.3, no.3, 1986, page 199 - 234, '' Gene, Vol. 30, No. 1052, 1984, pages 17-22; Elsevier Sc. Publ.; V.A. CORFIELD et al.: "A modified protoplast-regeneration protocol facilitating the detection of cloned exoenzyme genes in Bacillus subtilis" Biological Abstracts, Vol. 82, No. 9, 1986, page 871, Ref. No. 86427; Philadelphia, US, Y.J. HYUN et
al.: "Physiological properties and transformaties of alkaline-tolerant bacteria", & Korean J. Appl.

Microbiol. Bioeng, 14(3), 239-244, 1986 Agricultural and

Biological Chemistry, Vol. 47, No. 9, September 1983, pages 2101-2102; Tokyo, JP, R. USAMI et al.: "Plasmids in alkalophilic Bacillus sp." Proc. Natl. Acad. Sci. USA, Vol. 81. No. 4, February 1984, pages 1184-1188; Washington, US, S.-L. WONG et al.: "The subtilisin E gene of Bacillus subtilis is transcribed from a sigma-37

promoter in vivo", page 1187. Nature, Vol. 318, November

28, 1985, pages 375-376, P.G. THOMAS et al.: "Tailoring the pH dependance of enzyme catalysis using protein engineering", Figure 1. Biological Abstracts/RRM, Ref. No. 34060253; Philadelphia, US, C. PAK et al.: "Cloning of protease gene produced by heat-resistant alkalinic Bacillus in Escherichia coli", & Abstract Biotechnology Genetic Engineering, whole Abstract. Biological Abstracts/RRM, Ref. No. 85 003592; Philadelphia, US, B.C. CUNNINGHAM et al.: "Improvement in the alkaline stability of subtilisin using an efficient random mutagenesis and screening procedure", & Protein Eng 1987, 1, (4), 319-326, whole Abstract. Spec. Publ. Soc. Gen. Microbiol., Vol. 17, "Microbesd Extreme Environ", 1986, pages 297-315: chapter 10, K. HORIKOSHI: "Genetic applications of alkalophilic microorganisms", whole article

ABEN Novel methods and novel industrial **Bacillus** strains are provided for enhanced production of serine **protease**. Plasmid constructs comprising the serine **protease** gene are introduced into a compatible host, generally a **Bacillus** already overproducing serine **protease**. Preferred host cells are those from **Bacillus** novo sp. **PB92** and the preferred serine **protease** gene also originates from **Bacillus** **PB92**. Integration and maintenance of the plasmid in the host cell chromosome can be achieved by including a temperature sensitive origin of replication in the plasmid construct and growing under selective conditions.

L15 ANSWER 10 OF 11 EUROPATFULL COPYRIGHT 2001 WILA

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 220921 EUROPATFULL EW 198719 FS OS STA B
TITLE: Heat stable **alkaline proteases** produced by a **bacillus**.
Von einem **Bacillus** gebildete hitzebestaendige **alkalische Proteasen**.
Proteases alcalines stables a chaud produites par un **bacillus**.
INVENTOR(S): Stellwag, Edmund J., 1 Nickelby Court, Damascus Maryland

20872, US;
Swann, Wayne E., 5392 Storm Drift, Columbia Maryland 20906, US;
Stewart, David B., 1400 South Joyce Street Apt. A-1703, Arlington Virginia 22202, US;
Durham, Donald R., 20617 Beaver Ridge Road, Gaithersburg

Maryland 21045, US;
Nolf, Carol A., 14347 Rosetree Court, Silver Spring Maryland 20906, US
PATENT ASSIGNEE(S): GENEX CORPORATION, 16020, Industrial Drive, Gaithersburg

Maryland 20877, US

PATENT ASSIGNEE NO: 497132
AGENT: Holmes, Michael John, et al, Frank B. Dehn & Co. European Patent Attorneys Imperial House 15-19 Kingsway, London, WC2B 6UZ,, GB
OTHER SOURCE: ESP1987016 EP 0220921 A2 870506
SOURCE: Wila-EPZ-1987-H19-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
PATENT INFO.PUB.TYPE: EPA2 EUROPAEISCHE PATENTANMELDUNG
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 220921	**A2**	**19870506**

'OFFENLEGUNGS' DATE: 19870506
APPLICATION INFO.: EP 1986-308171 19861021
PRIORITY APPLN. INFO.: US 1985-790256 19851022
ABEN Novel enzymes exhibiting proteolytic activity in **alkaline** media and stability at high temperatures and under **alkaline** conditions are produced by a novel Ba.shy. cillus strain designated GX6638 or its **mutants** or var.shy. iants. These enzymes are especially well-suited for inclusion in washing compositions. A culture of GX6638 has been deposited with the American Type Cul.shy. ture Collection, Rockville, Maryland as ATCC No. 53278.

L15 ANSWER 11 OF 11 PATOSEP COPYRIGHT 2001 WILA

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 1988:1440341 PATOSEP ED 19920127 EW 198839 FS OS
TITLE: Stable gene amplification in prokaryotic chromosomal DNA.
Stabile Genamplifizierung in prokaryotischer chromosonaler DNA.
Amplification de genes stable dans l'ADN chromosomique des prokaryotes.
INVENTOR(S): Van Eekelen, Christiaan Albertus Gerardus, Bachplaats 14, NL-2661 HD Bergschenhoek, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1, NL-1062 CP Amsterdam, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL
PATENT ASSIGNEE(S): GIST-BROCADES N.V., Wateringseweg 1, NL-2611 XT Delft, NL
PATENT ASSIGNEE NO: 200380
AGENT: Huygens, Arthur Victor, Dr. et al, c/o Gist-Brocades N.V. Patent & Trademarks Department Wateringseweg 1 PO Box 1, NL-2600 MA Delft, NL
AGENT NUMBER: 20213
OTHER SOURCE: ESP1988035 EP 0284126 A1 0035
SOURCE: Wila-EPZ-1988-H39-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
PATENT INFO.PUB.TYPE: EPA1 EUROPAEISCHE PATENTANMELDUNG
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 284126	**A1**	**19880928**

'OFFENLEGUNGS' DATE: 19880928
APPLICATION INFO.: EP 1988-200376 19880229
PRIORITY APPLN. INFO.: EP 1987-200356 19870227

GRANTED PATENT - ERTEILTES PATENT - BREVET DELIVRE

ACCESSION NUMBER: 1988:1440341 PATOSEP UP 19930829 EW 199332 FS PS
TITLE: Stable gene amplification in prokaryotic chromosomal DNA.
Stabile Genamplifizierung in prokaryotischer chromosonaler DNA.
Amplification de genes stable dans l'ADN chromosomique des prokaryotes.
INVENTOR(S): Van Eekelen, Christiaan Albertus Gerardus, Bachplaats 14, NL-2661 HD Bergschenhoek, NL;
Van der Laan, Johannes Cornelis, J. Jongkindstraat 81/1,
NL-1062 CP Amsterdam, NL;
Mulleners, Leonardus Johannes Sofie Marie, Hoeksestraat 42, NL-5121 SV Rijen, NL
PATENT ASSIGNEE(S): GIST-BROCADES N.V., Wateringseweg 1, NL-2611 XT Delft, NL
PATENT ASSIGNEE NO: 200380
AGENT: Huygens, Arthur Victor, Dr. et al, c/o Gist-Brocades N.V. Patents & Trademarks Department Wateringseweg 1 PO Box 1, NL-2600 MA Delft, NL
AGENT NUMBER: 20213
OTHER SOURCE: EPB1993039 EP 0284126 B1 0049
SOURCE: Wila-EPS-1993-H32-T1
DOCUMENT TYPE: Patent
LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
DESIGNATED STATES: R AT; R BE; R CH; R DE; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
PATENT INFO.PUB.TYPE: EPB1 EUROPAEISCHE PATENTSCHRIFT
PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 284126	**B1**	**19930811**
'OFFENLEGUNGS' DATE:		19880928
APPLICATION INFO.: EP 1988-200376		19880229
PRIORITY APPLN. INFO.: EP 1987-200356		19870227
REFERENCE PAT. INFO.: EP 32238	A	EP 74553 A
EP 124374	A	EP 127328 A
EP 130756	A	EP 134048 A
EP 205371	A	WO 86-01825 A
WO 87-04461	A	FR 2292042 A

REF. NON-PATENT-LIT.: CHEMICAL ABSTRACTS, vol. 98, 1983, page 88, abstract no.
155686a, Columbus, Ohio, US; J. HOFEMEISTER et al.: "Integration of plasmid pE194 at multiple sites on the Bacillus subtilis chromosome"
CHEMICAL ABSTRACTS, vol. 108, 1988, page 180, abstract no. 88925n, Columbus, Ohio, US; P. KALLIO et al.: "Enhancement of alpha-amylase production by integrating and amplifying the alpha-amylase gene of Bacillus amyloliquefaciens in the genome of Bacillus subtilis"
BIOLOGICAL ABSTRACTS, vol. 82, 1986, abstract no. 86427,
Biosciences Information Service, Philadelphia, US; J.H. YU et al.: "Physiological properties and transformation of alkaline-tolerant bacteria"
GENE, vol. 24, 28th September 1983, pages 37-51, Elsevier Science Publishers, Amsterdam, NL; J.G.K. WILLIAMS et al.: "Stable integration of foreign DNA into
the chromosome of the cyanobacterium Synechococcus R2"

EPA1 EUROPAEISCHE PATENTANMELDUNG
EPB1 EUROPAEISCHE PATENTSCHRIFT
EPLS LEGAL STATUS
EPLU LEGAL STATUS, UPDATE

ABEN Transformed prokaryotic hosts are provided comprising two or more copies of a DNA sequence stably maintained in their chromosome, said sequence comprising a gene encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences. Methods are also provided for producing said transformed host strains. Said transformed host strains are capable of increased production of the polypeptide of interest compared to host strains which already produce said polypeptide. Preferred host strains are **Bacillus** novo species **PB92** which produces a high-**alkaline** proteolytic enzyme and **Bacillus** licheniformis T5 which produce a thermostable alpha-amylase, and **mutants** and variants of said strains. Preferred polypeptide encoding genes are the **protease** encoding gene originating from **Bacillus PB92** and the alpha-amylase encoding gene originating from **Bacillus** licheniformis strain T5.

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Search Results -

Terms	Documents
l13 and l3	25

Database: US Patents Full Text Database
JPO Abstracts Database
EPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Refine Search: l13 and l3 Clear

Search History

Today's Date: 3/10/2001

DB Name	Query	Hit Count	Set Name
USPT	l13 and l3	25	L14
USPT	l6 and l5	25	L13
USPT	l10 and l8 and l7 and l6	1	L12
USPT	l10 and l9 and l8 and l7 and l6	0	L11
USPT	(((536/23.1)!.CCLS.))	4778	L10
USPT	(((435/477)!.CCLS.))	125	L9
USPT	(((435/320.1)!.CCLS.))	8758	L8
USPT	(((435/252.31)!.CCLS.))	487	L7
USPT	((435/221)!.CCLS.)	193	L6
USPT	l4 and muta$	49	L5
USPT	l3 and l2 and l1	54	L4
USPT	pb92	69	L3
USPT	bacillus	16405	L2
USPT	protease and alkaline	10571	L1

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Search Results - Record(s) 1 through 49 of 49 returned.

☐ 1. Document ID: US 6197589 B1

L5: Entry 1 of 49 File: USPT Mar 6, 2001

US-PAT-NO: 6197589
DOCUMENT-IDENTIFIER: US 6197589 B1
TITLE: Enzymes for detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 6197567 B1

L5: Entry 2 of 49 File: USPT Mar 6, 2001

US-PAT-NO: 6197567
DOCUMENT-IDENTIFIER: US 6197567 B1
TITLE: Modified subtilisins and detergent compositions containing same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 3. Document ID: US 6190904 B1

L5: Entry 3 of 49 File: USPT Feb 20, 2001

US-PAT-NO: 6190904
DOCUMENT-IDENTIFIER: US 6190904 B1
TITLE: High-alkaline protease and its use arginine-substituted subtilisin composition and use

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 4. Document ID: US 6190900 B1

L5: Entry 4 of 49 File: USPT Feb 20, 2001

US-PAT-NO: 6190900
DOCUMENT-IDENTIFIER: US 6190900 B1
TITLE: Subtilase variants

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 5. Document ID: US 6165718 A

L5: Entry 5 of 49 File: USPT Dec 26, 2000

US-PAT-NO: 6165718
DOCUMENT-IDENTIFIER: US 6165718 A
TITLE: Method for in vivo production of a mutant library in cells

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 6. Document ID: US 6162260 A

L5: Entry 6 of 49 File: USPT Dec 19, 2000

US-PAT-NO: 6162260
DOCUMENT-IDENTIFIER: US 6162260 A
TITLE: Single-bath biopreparation and dyeing of textiles

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 7. Document ID: US 6140109 A

L5: Entry 7 of 49 File: USPT Oct 31, 2000

US-PAT-NO: 6140109
DOCUMENT-IDENTIFIER: US 6140109 A
TITLE: Method for enzymatic treatment of wool

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 8. Document ID: US 6136553 A

L5: Entry 8 of 49 File: USPT Oct 24, 2000

US-PAT-NO: 6136553
DOCUMENT-IDENTIFIER: US 6136553 A
TITLE: Mutant proteolytic enzymes and method of production

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 9. Document ID: US 6127144 A

L5: Entry 9 of 49 File: USPT Oct 3, 2000

US-PAT-NO: 6127144
DOCUMENT-IDENTIFIER: US 6127144 A
TITLE: Method for expression of proteins in bacterial host cells

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 10. Document ID: US 6124097 A

L5: Entry 10 of 49 File: USPT Sep 26, 2000

US-PAT-NO: 6124097
DOCUMENT-IDENTIFIER: US 6124097 A
TITLE: Stable gene amplification in chromosomal DNA of prokaryotic microorganisms

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 11. Document ID: US 6114509 A

L5: Entry 11 of 49 File: USPT Sep 5, 2000

US-PAT-NO: 6114509
DOCUMENT-IDENTIFIER: US 6114509 A
TITLE: Polypeptide with reduced allergenicity

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 12. Document ID: US 6099588 A

L5: Entry 12 of 49 File: USPT Aug 8, 2000

US-PAT-NO: 6099588
DOCUMENT-IDENTIFIER: US 6099588 A
TITLE: Method for treatment of wool

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 13. Document ID: US 6100080 A

L5: Entry 13 of 49 File: USPT Aug 8, 2000

US-PAT-NO: 6100080
DOCUMENT-IDENTIFIER: US 6100080 A
TITLE: Method for enzymatic treatment of biofilm

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 14. Document ID: US 6066473 A

L5: Entry 14 of 49 File: USPT May 23, 2000

US-PAT-NO: 6066473
DOCUMENT-IDENTIFIER: US 6066473 A
TITLE: Introduction of DNA into bacillus strains by conjugation

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 15. Document ID: US 6066611 A

L5: Entry 15 of 49 File: USPT May 23, 2000

US-PAT-NO: 6066611
DOCUMENT-IDENTIFIER: US 6066611 A
TITLE: Bleaching compositions comprising protease enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw Desc | Image

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☐ 16. Document ID: US 6051033 A

L5: Entry 16 of 49 File: USPT Apr 18, 2000

US-PAT-NO: 6051033
DOCUMENT-IDENTIFIER: US 6051033 A
TITLE: Method for enzymatic treatment of wool

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw Desc | Image

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☐ 17. Document ID: US 6017871 A

L5: Entry 17 of 49 File: USPT Jan 25, 2000

US-PAT-NO: 6017871
DOCUMENT-IDENTIFIER: US 6017871 A
TITLE: Protease-containing cleaning compositions

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw Desc | Image

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☐ 18. Document ID: US 5985639 A

L5: Entry 18 of 49 File: USPT Nov 16, 1999

US-PAT-NO: 5985639
DOCUMENT-IDENTIFIER: US 5985639 A
TITLE: Mutant proteolytic enzymes and method of production

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw Desc | Image

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☐ 19. Document ID: US 5981255 A

L5: Entry 19 of 49 File: USPT Nov 9, 1999

US-PAT-NO: 5981255
DOCUMENT-IDENTIFIER: US 5981255 A
TITLE: Alkaline protease, process for the production thereof, use thereof, and microorganism producing the same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw Desc | Image

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☐ 20. Document ID: US 5981718 A

Entry 20 of 49 File: USPT Nov 9, [illegible]

US-PAT-NO: 5981718
DOCUMENT-IDENTIFIER: US 5981718 A
TITLE: Polypeptide with reduced allergenicity

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 21. Document ID: US 5856451 A

L5: Entry 21 of 49 File: USPT Jan 5, 1999

US-PAT-NO: 5856451
DOCUMENT-IDENTIFIER: US 5856451 A
TITLE: Method for reducing respiratory allergenicity

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 22. Document ID: US 5843720 A

L5: Entry 22 of 49 File: USPT Dec 1, 1998

US-PAT-NO: 5843720
DOCUMENT-IDENTIFIER: US 5843720 A
TITLE: Introduction of DNA into bacillus strains by conjugation

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 23. Document ID: US 5837517 A

L5: Entry 23 of 49 File: USPT Nov 17, 1998

US-PAT-NO: 5837517
DOCUMENT-IDENTIFIER: US 5837517 A
TITLE: Protease variants and compositions

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 24. Document ID: US 5834299 A

L5: Entry 24 of 49 File: USPT Nov 10, 1998

US-PAT-NO: 5834299
DOCUMENT-IDENTIFIER: US 5834299 A
TITLE: Method for dehairing of hides or skins by means of enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 25. Document ID: US 5830740 A

L5: Entry 25 of 49 File: USPT Nov 3, 1998

US-PAT-NO: 5830740
DOCUMENT-IDENTIFIER: US 5830740 A
TITLE: Serine protease operative between 75.degree.C. and 103.degree.C.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 26. Document ID: US 5801039 A

L5: Entry 26 of 49 File: USPT Sep 1, 1998

US-PAT-NO: 5801039
DOCUMENT-IDENTIFIER: US 5801039 A
TITLE: Enzymes for detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 27. Document ID: US 5766898 A

L5: Entry 27 of 49 File: USPT Jun 16, 1998

US-PAT-NO: 5766898
DOCUMENT-IDENTIFIER: US 5766898 A
TITLE: Proteins with changed epitopes and methods for the production thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 28. Document ID: US 5733723 A

L5: Entry 28 of 49 File: USPT Mar 31, 1998

US-PAT-NO: 5733723
DOCUMENT-IDENTIFIER: US 5733723 A
TITLE: Stable gene amplification in chromosomal DNA of prokaryotic microorganisms

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 29. Document ID: US 5693602 A

L5: Entry 29 of 49 File: USPT Dec 2, 1997

US-PAT-NO: 5693602
DOCUMENT-IDENTIFIER: US 5693602 A
TITLE: Spray dried powered automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 30. Document ID: US 5679630 A

L5: Entry 30 of 49 File: USPT Oct 21, 1997

US-PAT-NO: 5679630
DOCUMENT-IDENTIFIER: US 5679630 A
TITLE: Protease-containing cleaning compositions

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 31. Document ID: US 5677272 A

L5: Entry 31 of 49 File: USPT Oct 14, 1997

US-PAT-NO: 5677272
DOCUMENT-IDENTIFIER: US 5677272 A
TITLE: Bleaching compositions comprising protease enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 32. Document ID: US 5665587 A

L5: Entry 32 of 49 File: USPT Sep 9, 1997

US-PAT-NO: 5665587
DOCUMENT-IDENTIFIER: US 5665587 A
TITLE: Modified subtilisins and detergent compositions containing same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 33. Document ID: US 5631217 A

L5: Entry 33 of 49 File: USPT May 20, 1997

US-PAT-NO: 5631217
DOCUMENT-IDENTIFIER: US 5631217 A
TITLE: Detergent compositions comprising a modified subtilisin

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 34. Document ID: US 5618465 A

L5: Entry 34 of 49 File: USPT Apr 8, 1997

US-PAT-NO: 5618465
DOCUMENT-IDENTIFIER: US 5618465 A
TITLE: Nonaqueous liquid automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 35. Document ID: US 5545344 A

L5: Entry 35 of 49 File: USPT Aug 13, 1996

US-PAT-NO: 5545344
DOCUMENT-IDENTIFIER: US 5545344 A
TITLE: Nonaqueous liquid, improved automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 36. Document ID: US 5527483 A

L5: Entry 36 of 49 File: USPT Jun 18, 1996

US-PAT-NO: 5527483
DOCUMENT-IDENTIFIER: US 5527483 A
TITLE: Nonaqueous gelled automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 37. Document ID: US 5510048 A

L5: Entry 37 of 49 File: USPT Apr 23, 1996

US-PAT-NO: 5510048
DOCUMENT-IDENTIFIER: US 5510048 A
TITLE: Nonaqueous liquid, phosphate-free, improved autoamatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 38. Document ID: US 5500364 A

L5: Entry 38 of 49 File: USPT Mar 19, 1996

US-PAT-NO: 5500364
DOCUMENT-IDENTIFIER: US 5500364 A
TITLE: Bacillus lentus alkaline protease varints with enhanced stability

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 39. Document ID: US 5482849 A

L5: Entry 39 of 49 File: USPT Jan 9, 1996

US-PAT-NO: 5482849
DOCUMENT-IDENTIFIER: US 5482849 A
TITLE: Subtilisin mutants

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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40. Document ID: US 5474699 A

L5: Entry 40 of 49 File: USPT Dec 12, 1995

US-PAT-NO: 5474699
DOCUMENT-IDENTIFIER: US 5474699 A
TITLE: Phosphate containing powered automatic dishwashing composition with enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 41. Document ID: US 5468411 A

L5: Entry 41 of 49 File: USPT Nov 21, 1995

US-PAT-NO: 5468411
DOCUMENT-IDENTIFIER: US 5468411 A
TITLE: Powdered automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 42. Document ID: US 5387518 A

L5: Entry 42 of 49 File: USPT Feb 7, 1995

US-PAT-NO: 5387518
DOCUMENT-IDENTIFIER: US 5387518 A
TITLE: Alkaline protease having stability in solution with anionic surfactant, method for producing the same, use thereof and microorganism producing the same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 43. Document ID: US 5340735 A

L5: Entry 43 of 49 File: USPT Aug 23, 1994

US-PAT-NO: 5340735
DOCUMENT-IDENTIFIER: US 5340735 A
TITLE: Bacillus lentus alkaline protease variants with increased stability

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 44. Document ID: US 5336611 A

L5: Entry 44 of 49 File: USPT Aug 9, 1994

US-PAT-NO: 5336611
DOCUMENT-IDENTIFIER: US 5336611 A
TITLE: PB92 serine protease muteins and their use in detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 45. Document ID: US 5324653 A

L5: Entry 45 of 49 File: USPT Jun 28, 1994

US-PAT-NO: 5324653
DOCUMENT-IDENTIFIER: US 5324653 A
TITLE: Recombinant genetic means for the production of serine protease muteins

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 46. Document ID: US 5316935 A

L5: Entry 46 of 49 File: USPT May 31, 1994

US-PAT-NO: 5316935
DOCUMENT-IDENTIFIER: US 5316935 A
TITLE: Subtilisin variants suitable for hydrolysis and synthesis in organic media

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 47. Document ID: US 5217878 A

L5: Entry 47 of 49 File: USPT Jun 8, 1993

US-PAT-NO: 5217878
DOCUMENT-IDENTIFIER: US 5217878 A
TITLE: Molecular cloning and expression of genes encoding proteolytic enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 48. Document ID: US 4771003 A

L5: Entry 48 of 49 File: USPT Sep 13, 1988

US-PAT-NO: 4771003
DOCUMENT-IDENTIFIER: US 4771003 A
TITLE: Heat stable alkaline proteases produced by a bacillus

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 49. Document ID: US 4764470 A

L5: Entry 49 of 49 File: USPT Aug 16, 1988

US-PAT-NO: 4764470
DOCUMENT-IDENTIFIER: US 4764470 A
TITLE: Alkaline protease produced by a bacillus

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

Generate Collection

Terms	Documents
l4 and muta$	49

Display 49 Documents, starting with Document: 49

Display Format: TI Change Format

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Search Results - Record(s) 1 through 25 of 25 returned.

☐ 1. Document ID: US 6197589 B1

L14: Entry 1 of 25 File: USPT Mar 6, 2001

US-PAT-NO: 6197589
DOCUMENT-IDENTIFIER: US 6197589 B1
TITLE: Enzymes for detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 6197567 B1

L14: Entry 2 of 25 File: USPT Mar 6, 2001

US-PAT-NO: 6197567
DOCUMENT-IDENTIFIER: US 6197567 B1
TITLE: Modified subtilisins and detergent compositions containing same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 3. Document ID: US 6190904 B1

L14: Entry 3 of 25 File: USPT Feb 20, 2001

US-PAT-NO: 6190904
DOCUMENT-IDENTIFIER: US 6190904 B1
TITLE: High-alkaline protease and its use arginine-substituted subtilisin composition and use

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 4. Document ID: US 6190900 B1

L14: Entry 4 of 25 File: USPT Feb 20, 2001

US-PAT-NO: 6190900
DOCUMENT-IDENTIFIER: US 6190900 B1
TITLE: Subtilase variants

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 5. Document ID: US 6136553 A

L14: Entry 5 of 25 File: USPT Oct 24, 2000

US-PAT-NO: 6136553
DOCUMENT-IDENTIFIER: US 6136553 A
TITLE: Mutant proteolytic enzymes and method of production

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 6. Document ID: US 5985639 A

L14: Entry 6 of 25 File: USPT Nov 16, 1999

US-PAT-NO: 5985639
DOCUMENT-IDENTIFIER: US 5985639 A
TITLE: Mutant proteolytic enzymes and method of production

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 7. Document ID: US 5981255 A

L14: Entry 7 of 25 File: USPT Nov 9, 1999

US-PAT-NO: 5981255
DOCUMENT-IDENTIFIER: US 5981255 A
TITLE: Alkaline protease, process for the production thereof, use thereof, and microorganism producing the same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 8. Document ID: US 5837517 A

L14: Entry 8 of 25 File: USPT Nov 17, 1998

US-PAT-NO: 5837517
DOCUMENT-IDENTIFIER: US 5837517 A
TITLE: Protease variants and compositions

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 9. Document ID: US 5801039 A

L14: Entry 9 of 25 File: USPT Sep 1, 1998

US-PAT-NO: 5801039
DOCUMENT-IDENTIFIER: US 5801039 A
TITLE: Enzymes for detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 10. Document ID: US 5766898 A

L14: Entry 10 of 25 File: USPT Jun 16, 1998

US-PAT-NO: 5766898
DOCUMENT-IDENTIFIER: US 5766898 A
TITLE: Proteins with changed epitopes and methods for the production thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 11. Document ID: US 5679630 A

L14: Entry 11 of 25 File: USPT Oct 21, 1997

US-PAT-NO: 5679630
DOCUMENT-IDENTIFIER: US 5679630 A
TITLE: Protease-containing cleaning compositions

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 12. Document ID: US 5677272 A

L14: Entry 12 of 25 File: USPT Oct 14, 1997

US-PAT-NO: 5677272
DOCUMENT-IDENTIFIER: US 5677272 A
TITLE: Bleaching compositions comprising protease enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 13. Document ID: US 5665587 A

L14: Entry 13 of 25 File: USPT Sep 9, 1997

US-PAT-NO: 5665587
DOCUMENT-IDENTIFIER: US 5665587 A
TITLE: Modified subtilisins and detergent compositions containing same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 14. Document ID: US 5631217 A

L14: Entry 14 of 25 File: USPT May 20, 1997

US-PAT-NO: 5631217
DOCUMENT-IDENTIFIER: US 5631217 A
TITLE: Detergent compositions comprising a modified subtilisin

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 15. Document ID: US 5527483 A

L14: Entry 15 of 25 File: USPT [illegible]8, 1996

US-PAT-NO: 5527483
DOCUMENT-IDENTIFIER: US 5527483 A
TITLE: Nonaqueous gelled automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 16. Document ID: US 5500364 A

L14: Entry 16 of 25 File: USPT Mar 19, 1996

US-PAT-NO: 5500364
DOCUMENT-IDENTIFIER: US 5500364 A
TITLE: Bacillus lentus alkaline protease varints with enhanced stability

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 17. Document ID: US 5482849 A

L14: Entry 17 of 25 File: USPT Jan 9, 1996

US-PAT-NO: 5482849
DOCUMENT-IDENTIFIER: US 5482849 A
TITLE: Subtilisin mutants

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 18. Document ID: US 5468411 A

L14: Entry 18 of 25 File: USPT Nov 21, 1995

US-PAT-NO: 5468411
DOCUMENT-IDENTIFIER: US 5468411 A
TITLE: Powdered automatic dishwashing composition containing enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 19. Document ID: US 5387518 A

L14: Entry 19 of 25 File: USPT Feb 7, 1995

US-PAT-NO: 5387518
DOCUMENT-IDENTIFIER: US 5387518 A
TITLE: Alkaline protease having stability in solution with anionic surfactant, method for producing the same, use thereof and microorganism producing the same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 20. Document ID: US 5340735 A

L14: Entry 20 of 25 File: USPT Aug 23, 1994

US-PAT-NO: 5340735
DOCUMENT-IDENTIFIER: US 5340735 A
TITLE: Bacillus lentus alkaline protease variants with increased stability

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 21. Document ID: US 5336611 A

L14: Entry 21 of 25 File: USPT Aug 9, 1994

US-PAT-NO: 5336611
DOCUMENT-IDENTIFIER: US 5336611 A
TITLE: PB92 serine protease muteins and their use in detergents

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 22. Document ID: US 5324653 A

L14: Entry 22 of 25 File: USPT Jun 28, 1994

US-PAT-NO: 5324653
DOCUMENT-IDENTIFIER: US 5324653 A
TITLE: Recombinant genetic means for the production of serine protease muteins

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 23. Document ID: US 5217878 A

L14: Entry 23 of 25 File: USPT Jun 8, 1993

US-PAT-NO: 5217878
DOCUMENT-IDENTIFIER: US 5217878 A
TITLE: Molecular cloning and expression of genes encoding proteolytic enzymes

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 24. Document ID: US 4771003 A

L14: Entry 24 of 25 File: USPT Sep 13, 1988

US-PAT-NO: 4771003
DOCUMENT-IDENTIFIER: US 4771003 A
TITLE: Heat stable alkaline proteases produced by a bacillus

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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☐ 25. Document ID: US 4764470 A

L14: Entry 25 of 25 File: USPT Aug 16, 1988

US-PAT-NO: 4764470
DOCUMENT-IDENTIFIER: US 4764470 A
TITLE: Alkaline protease produced by a bacillus

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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Terms	Documents
l13 and l3	25

Display 25 Documents, starting with Document: 25

Display Format: TI Change Format